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THE UNIVERSITY OF ALBERTA

DIVERSITY OF LEUKOCYTE COMMON ANTIGEN (CD45) EXPRESSION

BY

RAYMOND KAI CHI LAI ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Medical Sciences

DEPARTMENT OF LABORATORY MEDICINE
AND PATHOLOGY

Edmonton, Alberta

Spring 1995

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Abstract

Leukocyte common antigen (LCA/CD45) is a family of transmembraneous molecules expressed exclusively on nucleated hemopoietic cells. Structural heterogeneity of LCA is largely confined to the extracellular domain and is generated by alternate usage of three exons of the LCA gene, commonly labeled variable region A, B and C. The cytoplasmic domain, that contains tyrosine phosphatase activity, is relatively constant.

We employed a panel of anti-LCA monoclonal antibodies to study the tissue distribution of LCA variants. The LCA extracellular domain was found to be far more heterogeneous than previously known. Our studies indicate that the total number of LCA isoforms expressed on lymphocytes is seven, by demonstrating the existence of two new LCA isoforms. We also observed extensive carbohydrate variation within the variable regions, creating an additional level of structural heterogeneity.

LCA differential glycosylation is not a random process, since the expression of the carbohydrate-related epitopes we have studied are specific for individual LCA isoforms and/or cell types. These results suggest that LCA glycosylation is functionally important, possibly by directing the interactions with multiple ligands and thus modulating the cytoplasmic tyrosine phosphatase activity.

Moreover, LCA epitopes which show cell-type specific expression may serve as markers for delineating different functional lymphoid subsets. In fact, using these reagents, we could demonstrate the existence of several distinct lymphoid subsets. Since these lymphoid subsets arise in postnatal life, they represent products of antigenic experience and/or maturation of the immune system. One example is a B-cell subset which expresses a high level of a carbohydrate-related epitope in the variable region B. The hypothesis that this B cell subset represents antigen-experienced/memory B cells, is supported by results from immunophenotypic and functional studies.

In summary, LCA expression is heterogeneous at the levels of protein backbone and of glycosylation. Lymphoid cells with different functions have unique patterns of LCA isoform and carbohydrates expression. This information facilitates further research on the function of LCA. Furthermore, the analysis of LCA expression with specific antibodies allows delineation of functional lymphoid subsets in various clinical settings.

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ABBREVIATIONS

kd	Kilodalton
LCA	Leukocyte common antigen
Mab	Monoclonal antibody
Fig	Figure
Ig	Immunoglobulin
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PMA	Phorbol ester
PHA	Phytohemagglutinin
mRNA	Messenger ribonucleic acids
MW	Molecular weight
CD	Cluster designation
SDS-PAGE	Sodium dodecyl sulphate-Polyacryamide gel electrophoresis

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

1. Background

The leukocyte common antigen (LCA, T200, CD45) family is a group of high molecular weight glycoproteins of around 200 kd uniquely expressed on the surface of hematopoietic cells [reviewed in 1]. LCA initially attracted attention in the early 1980s because of its abundance on lymphocytes, distinctive tissue distribution and novel structural features. The LCA family is a major cell surface component of lymphocytes and carries 10% of the total carbohydrates on the surface of these cells [2]. Furthermore, LCA is found only in the hemopoietic lineages. These phenomena raised the possibility that LCA may carry important functions in these cell types.

2. Structure of LCA

The LCA family of proteins is encoded by a single gene, mapped to chromosome 1 (1q31-32) in humans [3]. The gene is composed of 33 exons, three of which (exon 4, 5, and 6) are differentially expressed via alternate splicing of mRNA [4]. The regulation of the alternate splicing is not known.

The complete amino acid sequence of LCA was derived from isolated cDNA clones [5]. All LCA isoforms follow the same stuctural scheme: a large cytoplasmic domain of 707 amino acids encoded by exon 17 to 32, a trans-membrane region of 22 amino acids encoded by exon 16, and an external domain that varies between 391 to 552 amino acids, depending on the pattern of exon splicing used. Ultra-structurally, LCA was shown to consist of a globular structure representing the cytoplasmic domain and a rod-shaped structure representing the external domain [6].

The cytoplasmic domain is highly conserved among mammals and consists of at least two homologous subdomains, encoded by exons 17 to 24 and 25 to 32. The external domain is less conserved and includes an O-linked region and two cysteine-rich subdomains [1]. The two cysteine clusters are heavily glycosylated, located close to the trans-membraneous portion of the molecule, and are separated from each other by a loop structure. The O-linked region which is also heavily glycosylated spans the three so-called 'variable regions' (commonly labelled A, B and C) which are encoded by exon 4, 5 and 6 (fig. 1). Antigenic determinants unique to each of these three variable regions are labeled accordingly as CD45RA, CD45RB and CD45RC [7]. As alternate splicing of mRNA involves these three exons, variable regions are differentially expressed, creating different LCA isoforms (fig. 2). In humans, cDNA for a total of five different LCA isoforms were identified: they are ABC, AB, BC, B, with the fifth one lacking any of the three variable region (ie. O) [8,9].

Using SDS-electrophoresis coupled with immunoblotting, LCA proteins can be separated into four distinct bands at 220, 205, 190 and 180 kd. Streuli et al [10] showed that the molecular weights of LCA can be predicted on the basis of the number of variable regions expressed. Therefore, the 220 kd species corresponds to the LCA isoform expressing all three variable regions (ie. ABC), and the 180 kd species corresponds to the LCA isoform expressing none of the variable regions. The 190 kd species and 205 kd correspond to the LCA isoforms with one or two variable regions expressed, respectively.

3. LCA Cluster Antibodies

Antigenic epitopes located in the variable regions are termed 'restricted CD45' or CD45R, whereas those outside the variable regions are labelled CD45 [6]. Anti-CD45R Mab's are further specified according to the particular variable region they recognize. Therefore, anti-CD45RA, -CD45RB and -CD45RC are Mab's that recognize variable regions A, B and C respectively. There are antigenic epitopes unique to the 'O' isoform which are expressed only in the absence of all three variable regions. Mab's reactive with these epitopes are collectively labeled anti-CD45RO.

Studies of LCA have been hampered by the lack of Mab's reactive with some variable regions such as CD45RC. In the Leukocyte Workshop IV in 1989, there was only one anti-CD45RB Mab [9] and one anti-CD45RO Mab [10, 11] available. Furthermore, although there were a large number of anti-CD45RA Mab's available, they did not differ from each other significantly [6]. This limited number of anti-CD45R Mab's allowed only the detection of the variable regions on the cell surface but did not provide information as to the exact expression profile of LCA isoforms on the cells under study.

4. Tissue Distribution of LCA Isoforms

LCA is restricted to the hemopoietic cell lineage. In the early 1980s, it was found that members of LCA family are differentially expressed. Generally, B cells have LCA proteins of higher molecular weights at 220 kd and 205 kd. In contrast, T cells have LCA proteins of lower molecular weights at 180 kd and 190 kd [12-14].

While the LCA expression on B lymphocytes is relatively simple, the expression of LCA on T lymphocytes was found to be more complex. Expression of different LCA isoforms seems to be closely related to their functional and developmental stages. Immature thymocytes express only the 180 kd isoform (ie. CD45RO$^+$) [16]. As maturation occurs, LCA isoforms of higher molecular weight including the 220 kd and 205 kd species are expressed. At the same time, the 180 kd isoform is downregulated. These mature but antigen-inexperienced T cells are hence called CD45RA$^+$ T cells as they express largely the 220 kd (ABC) and 205 kd (AB) isoforms.

The expression of CD45RA and CD45RO is largely mutually exclusive on peripheral blood T cells. Tissue culture studies have shown that CD45RA$^+$ T cells switch their LCA expression to CD45RO and concurrently downregulate CD45RA expression upon antigenic stimulation [17,18]. These CD45RO$^+$ T cells also have a higher level of expression of lymphocyte activation markers including CD29, LFA-1 and MHC class II, as well as homing receptors such as CD44 [19]. CD45RO$^+$ T cells also seem to be more responsive to stimulation with low concentrations of anti-CD3 antibody than CD45RA$^+$ T cells [20, 21]. Cord blood T cells are largely CD45RA$^+$ [22, 23]. With age, the proportion of CD45RA$^+$ T cells decreases whereas that of CD45RO$^+$ T cells increases [24]. Based on these findings, it was proposed that CD45RA$^+$ and CD45RO$^+$ are markers for naive and memory T cells respectively [reviewed in 25].

Parellel to these studies, results from various laboratories suggest that at least some T cells can retain CD45RA expression after activation and these activated CD45RA$^+$ T cells produce different lymphokines from CD45RO$^+$ T cells [26, 27]. Therefore, some CD45RA$^+$ and CD45RO$^+$ T cells also represent two distinct lines of differentiation with different functions [28-30].

The highly regulated LCA expression in accordance with cellular differentiation and activation implies that the expression pattern of LCA isoforms is functionally important.

5. Functions of LCA

Early functional studies employed various immunologic assays in order to detect functional effects of anti-CD45/ anti-CD45R Mab's [31]. The results were sometimes contradictory and dependent on the Mab's employed. More recently, the use of LCA-deficient cell lines has provided more insight into the functional role(s) of LCA. T-cell clones deficient in the expression of LCA have impaired responses to T-cell receptor stimuli [32, 33]. Therefore, LCA appears to play an important role in early lymphocyte activation.

The biochemical basis of the functional role of LCA is linked to the two cytoplasmic homologous subdomains which have tyrosine phosphatase activity [34-36]. In fact, LCA serves as a prototype for a family of protein tyrosine phosphatases, some of which are transmembranous and share similar structure including the presence of tandem cytoplasmic enzymatic subdomains [37]. Recent studies demonstrated that some of the protein tyrosine kinases, such as $p56^{lck}$ and $p59^{fyn}$ proteins, are the physiological substrates of CD45 in T cells [38-40].

The expression of LCA seems to regulate phosphotyrosine homeostasis and to be linked to T-cell receptor-driven phosphoinositide hydrolysis and intracellular calcium increase after stimulation in T cells [41]. LCA may exert its biological effect via dephosphorylating protein tyrosine kinases such as $p56^{lck}$ and $p59^{fyn}$, or CD3 zeta chain. T cell activation via CD2 is also LCA-dependent [41].

LCA is also implicated in B cell activation. The B-cell antigen receptor can transduce a calcium-mobilizing signal only if cells also express LCA [42]. In addition, LCA can dephosphorylate a complex of membrane immunoglobulin associated proteins that appears to transduce signals [43]. Therefore, by regulating the phosphorylation status on tyrosine residues, LCA facilitates signal transduction.

7. The purpose of this study

LCA has crucial functional roles in lymphocyte activation by virtue of its tyrosine phosphatase activity which can dephosphorylate a number of important proteins in lymphocytes. However, how the LCA enzymatic activity is regulated is largely unknown.

It has been hypothesized that the control lies in the extracellular domain which may function as a receptor for one or more unknown ligands. The structural variation among the external domains of LCA probably allows for interaction with a variety of specific ligands and varies between different cell types. This hypothesis would explain why cells with different functions (eg. T vs B cells) express different LCA species. Features such as differential usage of the variable regions may certainly allow multiple ligand-receptor interactions, such that the LCA enzymatic activity can be directed to the appropriate targets at the right time.

Another issue is the high carbohydrate content in the extracellular domain, particularly the variable regions. One tempting notion is that LCA interaction with its ligand(s) is highly dependent on the glycosylation state. Because only a maximum of eight LCA species can be generated by alternate usage of three variable regions, it would be advantageous to create more structural variations, and therefore higher adaptability with a wide range of ligands expressed on different cell types.

With these issues and hypotheses, the following will be addressed in the later chapters:

1. By using a panel of monoclonal antibodies reactive with either the protein or carbohydrate epitopes on the extracellular domain, we explored further structural variations, in particular individual variable regions.

2. The expression patterns of individual LCA family members expressed on cells of different functions or developmental stages were also examined. A high tissue specificity is expected if these variations in glycosylation are functionally significant.

3. Another line of investigation is to examine if the LCA structural variations are acquired prenatally or postnatally. LCA expression on cord blood lymphocytes which are immunologically naive was compared with that of pediatric or adult peripheral lymphocytes. Tracing the evolution of specific LCA variations may provide clues to their possible functional roles.

4. As mentioned above, LCA has been valuable in delineating lymphoid subsets such as $CD45RA^+$ naive T cells and $CD45RO^+$ memory T cells. Exploring further LCA structural variations provides an opportunity to identify distinct lymphoid subsets with different functions or in distinct developmental stages.

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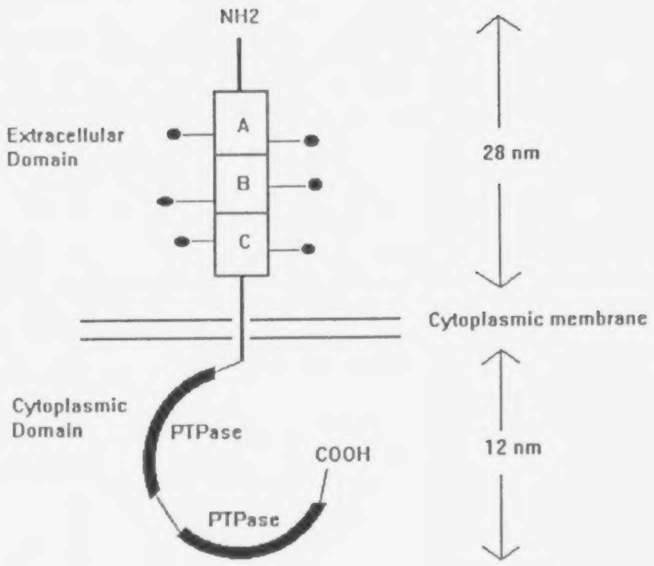
NH2
Extracellular Domain
A
B
C
28 nm
Cytoplasmic membrane
Cytoplasmic Domain
PTPase
COOH
12 nm
PTPase

Fig.1
Structure of LCA showing extracellualar, membraneous and cytoplasmic domain. The cytoplasmic domain contains two homologous regions with tyrosine phophatase activity (PTPase). On top of the the three variable regions (A, B and C) are O-linked glycosylation, illustrated as black circles.

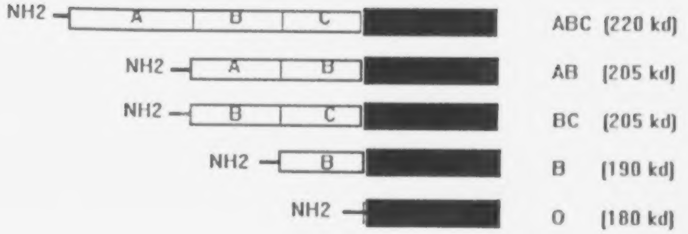
NH2
A
B
C
ABC (220 kd)
NH2
A
B
AB (205 kd)
NH2
B
C
BC (205 kd)
NH2
B
B (190 kd)
NH2
0 (180 kd)

Fig.2
Alternate splicing of mRNA leading to differential uasge of the three variable regions (A, B and C). Five LCA isoforms have been known to exist. The molecular weights of LCA isoforms correlate with the number of variable region expressed.

CHAPTER 2

TISSUE DISTRIBUTION OF RESTRICTED LEUCOCYTE COMMON ANTIGENS. A COMPREHENSIVE STUDY WITH PROTEIN AND CARBOHYDRATE SPECIFIC CD45R ANTIBODIES.

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INTRODUCTION

Leucocyte Common Antigen (LCA / T200 / CD45) is a family of cell surface glycoproteins found exclusively in nucleated cells of the hematopoietic lineage [1,8,10,11,21]. In humans, at least four LCA isoforms differing in molecular weight have been identified: they are the 180, 190, 205 and 220 kd isoforms [10,11,20,21]. Structurally, LCA has a well conserved cytoplasmic domain which is known to have tyrosine phosphatase activity [6,7,41], as well as an extracellular domain which is heterogenous in peptide sequence [15,29] and carbohydrate composition [2,18,28]. While little is known about the LCA carbohydrate component, the differential LCA peptide expression has been clearly delineated. Molecular studies of LCA have shown that alternate splicing of mRNA generates the heterogeneity of LCA [16,34,38]. The result is that LCA isoforms differ in the expression of the peptide domains encoded by the three alternate exons 4, 5 and 6. Streuli et al [36] proposed to designate these as A, B, and C, and at the Fourth Leucocyte Antigen Workshop it was decided to distinguish CD45RA, CD45RB, and CD45R0 antibodies (see Table 1). Antibodies recognizing common determinants of LCA (anti-CD45 or pan-leukocyte) are reactive with all four LCA isoforms. Antibodies recognizing a determinant on exon 7 that can only be detected in the absence of expression of all three variable exons are designated CD45R0. The reagents used in this study were analysed on LCA transfectants to confirm or establish their reactivity with common or specific variable regions of the leucocyte common antigen complex. Several studies included data on the tissue distribution of CD45R antigens [4,5,14,27]. Here we elaborate on these studies by including a series of anti-LCA antibodies which are either new or previously incompletely reported, to provide a comprehensive picture of CD45R distribution.

Materials and Methods

1. Monoclonal Antibodies

Anti-CD45RA Mabs MB1 and MT2, as well as anti-CD45RB Mab MT3, were produced in our laboratory and their specificities were recently delineated with the use of LCA transfectants. Anti-CD45RB Mab PD7/26 and anti-CD45

Mab 2B11 were kindly provided by Dr. D.Y. Mason. Anti-CD45 Mab 2D1 (HLe-1) was purchased from Becton Dickinson. Anti-CD45RO Mab UCHL-1 was kindly supplied by Dr. P. Beverly. KiB3 (CD45RA) was a gift from Dr. M. Parwaresch. The OKT3 and OKT11 cell lines were obtained from the American Type Culture Collection, Rockville, Maryland. Antibodies B-ly1 (CD20) and B-ly6 (CD11c) were produced in our laboratory.

2. Tissue Sections

Normal thymus tissue, removed for technical reasons during cardiac surgery, and tonsils and lymph nodes submitted for diagnostic reasons, were either B5-fixed and embedded in paraffin or fresh frozen in OCT. Frozen tissue sections (5 um) were cut using a Leitz cryostat. Paraffin sections were cut at 3 um.

3. Immunohistochemistry and enzymatic treatment

Paraffin-embedded tissue sections were deparaffinized with xylene and graded alcohols. Frozen section were fixed in acetone for 15 minutes. Five-fold dilutions of the different Mab's (supernatants) in PBS (pH 7.5) were then applied to the fixed paraffin or frozen sections and incubated for 30 minutes. After 3 washings with PBS, sections were treated with rabbit anti-mouse immunoglobulin conjugated with peroxidase (Jackson Immunoresearch). Peroxidase staining was obtained with a mixture of hydrogen peroxidase and 3-amino-9-ethylcarbazole (Sigma, St Louis, USA). To achieve double stainings, the first step was done as described above except that peroxidase staining was achieved with a mixture of H_2O_2 (Sigma) and diaminobenzidine (Sigma). After three washings with PBS, tissue sections were incubated with a five-fold dilution of the second Mab's (supernatants) for 30 minutes at room temperature, followed by alkaline phosphatase conjugated goat anti-mouse antibody (Dakopatts). Alkaline phosphatase enzyme staining was done by using a mixture of 60 mg of fast blue (Gurr), 10 mg of naphthol AS-MX phosphate (Sigma) and drops of 10% $MgSO_4$ (BDH chemicals). To test the neuraminidase sensitivity, tissue sections were treated with 10 micro-units neuraminidase type X (Sigma) per 0.1 mL PBS at 37 C for 15 minutes, and subsequently immunostained as described above. To remove O-linked carbohydrates, slides were first treated with neuraminidase (10 milli-unit per 0.1 mL of PBS) at 37 C for 15 minutes, followed by O-glycanase (Endo-alpha-N-acetyl-galactosaminidase) obtained from Boehringer

Mannheim at a concentration of 2 milli-units per 0.1 mL of PBS at 37 C for 30 minutes. Subsequent stainings were done as described above. To remove N-linked carbohydrates, slides were again treated with neuraminidase as described above, followed by N-glycanase 3 (Endoglycosidase F) obtained from Boehringer Mannheim at a concentration of 2 milli-unit per 0.1 mL of PBS at 37 C for 30 minutes. The effect of desialylation was examined as follows: slides were incubated with an antibody (for instance MB1), and with peroxidase conjugated rabbit anti-mouse antibody, and stained with diaminobenzidin and H2O2. After neuraminidase treatment as described above, the slides were incubated with the same primary antibody (for instance MB1), followed by alkaline phosphatase conjugated goat anti-mouse antibody and alkaline phosphatase enzyme staining. Any cells staining blue for the second procedure were interpreted as expressing antigenic epitopes that could not be detected before the desialylation.

4. Cell isolation and enrichment for lymphocytes subsets

Peripheral blood was collected from healthy individuals and mononuclear cells were isolated using ficoll-paque (Pharmacia). To enrich T cells, the mononuclear cells were incubated with Mab Bly-1 (CD20) at 4 C for 30 minutes. After 3 washings with PBS, B cells were depleted by using anti-mouse Ig coated magnetic beads (Dynabeads M450) from Dynal. Enrichment for B cells was done by sheep red blood cell rosetting. Further depletion was achieved by using a mixture of OKT11 (CD2) and OKT3 (CD3) and anti-mouse Ig coated magnetic beads.

5. Immunofluorescence stainings and Flow cytometry

After the treatment of primary Mab's at 4 C for 30 minutes, goat anti-mouse Mab coupled with phycoerythrin (Becton Dickinson) was applied for another 30 minutes at 4 C. Gating based on size and forward scatter, along with the cell enrichment, resulted in more than 96% purity in monocytes, 98% in T cells and 92% in B cells. Purity was assessed by using cell-type specific Mab's: Bly-6 (monocytes), Bly-1 (B cells) and OKT3 + OKT11 (T cells).

RESULTS AND DISCUSSION

We collected a panel of monoclonal antibodies that were produced in our laboratory and by several other groups and are well defined with respect to their specificities for constant or variable regions of the leucocyte common antigen complex. Standard reagents, such as 2D1 (CD45), 2H4 (CD45RA), PD7/26 (CD45RB) and UCHL1 (CD45RO) were studied and compared to a series of other less well defined antibodies, that were selected based on at least three of the following criteria: differences in staining patterns with the standard reagents, reactivity with molecular weights around 200 kd, reactivity with LCA transfectants, and clustering within CD45 or CD45R in one of the Leucocyte Antigen Workshops. These antibodies were used as probes to analyse the expression pattern of LCA variants on human lymphoid tissues from different anatomical compartments employing immunohistochemical and flow cytometric techniques. This also provided the opportunity to compare antibodies reactive with antigenic determinants encoded by the same variable peptide region but leading to differences in staining pattern. Since one of the possible explanations involved the presence of differences in carbohydrate composition, the effects of a partial removal of carbohydrates with different enzymes was studied.

1. Determination of carbohydrate reactivity of the antibodies

The determination of the biochemical nature of the epitopes recognized by the monoclonal antibodies (Mab's) was performed on frozen as well as on paraffin sections of reactive lymph nodes. The results are summarized in table 2. With the exception of 2D1 (CD45), and 2H4, MB1 and KiB3 (CD45RA), all the other CD45R/CD45 Mab's recognized neuraminidase sensitive epitopes. This finding suggests that the epitopes they recognize are associated with carbohydrates, and very likely, with the terminal sialic acids commonly found on cell surface glycoproteins. On the other hand, 2D1, 2H4, MB1 and KiB3 are probably reactive with the LCA peptide backbone. The expression of the MT2 determinant (CD45RA) on mantle zone B cells was more resistant to neuraminidase treatment than that on the paracortical T lymphocytes and other B cells, suggesting a unique glycosylation of CD45RA on mantle zone B cells. PD7/26 (CD45RB) was more resistant to neuraminidase treatment than the other neuraminidase- sensitive mab's including MT3 (also CD45RB). We also incubated slides with neuraminidase and subsequently with O-glycanase to remove O-linked carbohydrates. No detectable change of staining for MB1 or 2H4 (CD45RA) was observed; however, slides stained with KiB3 (also CD45RA) showed additional staining, leading to a pattern identical to that of other anti-CD45RA Mab's (see below). Removal of N-linked carbohydrates using neuraminidase followed by N-glycanase did not result in detectable change in MB1 or KiB3 staining.

2. Tissue distribution

The results of the distribution studies with the CD45/CD45R panel of reagents are summarized in table 2. A detailed analysis of the staining results in thymus, lymph nodes/tonsils, spleens and peripheral blood cells is given below.

2a. Thymus

In agreement with the view that LCA is one of the earliest antigens expressed by lymphocytes, all cells with the exception of a small subpopulation of subcortical thymocytes, were 2D1 and 2B11 (CD45) positive (fig 1a). There was no detectable difference in the intensity of staining between medullary and cortical thymocytes. The majority of thymocytes were also MT3 and PD7/26 (CD45RB) positive (fig 1b), indicating the presence of CD45RB on the cell surface. Unlike the anti-CD45 antibodies, the anti-CD45RB Mab's resulted in differences in staining intensity, with cells in the cortex staining less strong than those in the medulla. This probably reflects the fact that anti-CD45 stains all LCA, including the 180 kd isoform, whereas anti-CD45RB reagents do not stain the 180 kd isoform that is strongly expressed on cortical thymocytes [37]. CD45RA positive cells as identified by 2H4, MB1, KiB3 and MT2 were largely confined to the medulla with only a few positive cells in the cortex (fig 1c). MT2 (also CD45RA) was reactive with more thymocytes than the other anti-CD45RA reagents. Double staining with MB1 and MT2 showed the presence of $MT2^{+}MB1^{-}$ but not $MT2^{-}MB1^{+}$ thymocytes. Since MT2 reacts with a carbohydrate component associated with CD45RA, whereas MB1 and 2H4 react with the peptide backbone (see above), one likely explanation for this phenomenon would be that the MT2 carbohydrate epitope in fact masks the binding sites for MB1 and 2H4 on these cells. To test this possibility, we stained sections with MB1 or 2H4, peroxidase labeled secondary antibodies and diaminobenzidin, and then the sections were treated with neuraminidase. Subsequently we stained the sections again with MB1 or 2H4, alkaline phosphatase labeled secondary antibodies and naphthol AS MX sulphate and fast blue. This resulted in the additional staining of cells for MB1 or 2H4 in medulla and cortex, leading to a combined pattern identical to the MT2 staining (fig 2a,b). This finding indicates that, within a subpopulation of the thymocytes (ie. MB1$^-$MT2$^+$ cells), the MB1 and 2H4 epitopes are masked by the presence of terminal sialic acids associated with the CD45RA region. KiB3 (also CD45RA) was reactive with an even smaller number of cells than MB1 and 2H4. In fact, KiB3 only recognized medullary B lymphocytes. Pretreatment of the sections with either neuraminidase or neuraminidase followed by N-glycanase did not result in any change. However, pretreatment with neuraminidase followed by O-glycanase led to a staining pattern of KiB3 that was identical to that of the other anti-CD45RA mab's. This finding indicates that the presence of O-linked carbohydrates on LCA can block the binding sites for anti-CD45RA antibodies that are reactive with the peptide backbone. This is in agreement with the finding that variable regions of LCA are rich in O-linked carbohydrates [39]. CD45R0 as recognized by Mabs UCHL-1, A6, and OPD4 had a reciprocal expression pattern with CD45RA. Virtually all cortical thymocytes as well as a small percentage of medullary thymocytes were positive for UCHL-1 (fig 1d). This reciprocal relationship was confirmed by a double staining method with these two groups of Mab's. The findings indicate that cortical thymocytes express CD45R0 and CD45RB molecules (180 and 190 kd), whereas medullary thymocytes express mostly CD45RA and CD45RB molecules (220, 205 and 190 kd). The significance of these differences in LCA composition of the thymocyte surface remains largely unclear. However, it has been suggested that LCA on thymocytes plays a role in thymic education [12,22].

2b. Lymph nodes and Tonsils

2D1 (CD45) was reactive with virtually all cells. The anti-CD45R Mab's included in the study, as well as anti-CD45 antibody 2B11 demonstrated a restricted reactivity pattern. The reactivities of the anti-CD45RA Mab's had unique features. With the exception of KiB3, they were reactive with B lymphocytes and a subset of T cells in the paracortex (fig 3a,b). While 2H4 and MB1 were positive in mantle zone and germinal center, MT2 was only positive in the mantle zone (fig 3c). As shown above, the MT2 epitope is the only CD45RA sensitive to neuraminidase treatment. It is known that mouse B cells will become hyposialylated upon IL-2 stimulation and that LCA is involved in that process [9]. The absence of the MT2 epitope from germinal center cells suggests a similar desialylation process for germinal center cells. This notion is supported by the finding that anti-CD45 mab 2B11 also has only very weak reactivity with germinal center cells [43] (fig.3d). This indicates that glycosylation outside of the variable regions may also vary. In accordance with the above findings, peanut agglutinin, which has binding sites that can be covered by terminal sialic acid, binds strongly to germinal center cells and not to mantle zone cells [32]. Recently we have found that peanut agglutinin reactivity on germinal center cells is largely the result of binding to LCA [not shown]. KiB3 (CD45RA) was not reactive with T cells in the paracortex, and although it did react with most B cells in mantle zone and germinal center, the percentage of positive cells in the mantle zone was around 70%, in contrast to the other anti-CD45RA Mab's that stained all mantle zone B cells (fig 4a). Pretreatment of the sections with neuraminidase followed by O-glycanase again resulted in a staining pattern for KiB3 that was identical to that of the other anti-CD45RA Mab's (fig 4b). While T cells in the paracortex were strongly MT3 (CD45RB) positive, B cells in the germinal center were weakly positive and only a minority of mantle zone B cells - up to 30% - were reactive with MT3. A subpopulation of strongly MT3 positive small T lymphocytes was present within the germinal center (fig 5a). In contrast, another anti-CD45RB antibody, PD7/26 was much less restricted in expression than MT3. Besides being positive in the paracortex, PD7/26 also reacted with B cell areas, including mantle zone and germinal center (fig 5b). This discrepancy can be explained by the fact that MT3 recognizes a carbohydrate group associated with CD45RB that is not expressed on all LCA with peptides encoded by the B exon. For instance, only transfectants expressing peptides encoded by exons AB or B, and not those expressing ABC or BC carry the MT3 determinant [27]. CD45R0 reactivity was confined to the CD45RA negative population: a majority of cells were staining in the paracortex, but UCHL-1 (CD45R0) was largely negative in the mantle zone and germinal center except for T cells clustered in the light zone, mostly at the border of the germinal center and the mantle zone (fig 5c). Double staining using UCHL-1 and MB1, showed that CD45R0$^+$ and CD45RA$^+$ cells in the paracortex area were also largely exclusive to each other, consistent with other studies showing that these are functionally different lymphocyte subsets [17, 33].

2c. Spleen

Within the white pulp of the spleen, there was a significant degree of diversity of CD45R expression. First, as in the lymph nodes/tonsils, MT2 differed from MB1 and 2H4 (all anti-CD45RA) in that it was not reactive with the germinal center B cells. Mantle zone B cells were positive for anti-CD45RA Mab's 2H4, MB1, and MT2. KiB3 (another CD45RA) stained the majority of mantle zone cells, but not the marginal zone B cells (fig 6a), whereas the other three anti-CD45RA mab's were positive on marginal zone cells as well as on 30% of cells in the periarteriolar lymphocyte sheath (fig 6d). The reactivities of KiB3 (CD45RA) and MT3 (CD45RB) were found to be reciprocal. Besides its strong reactivity with T cells, MT3 was also reactive with the marginal zone B cells (fig 6c). In contrast, KiB3 was reactive with the germinal center and mantle zone but not the marginal zone and T cells. A double immunostaining with these two Mab's confirmed that their expression was indeed exclusive to each other. Removal of O-linked but not N-linked carbohydrates resulted in a KiB3 staining pattern similar to that of the other anti-CD45RA mab, including the positivity with marginal zone cells (fig 6b). Although the functional significance of marginal zone B cells is unclear, the specific fine regulation of KiB3/MT3 expression may indicate a role of LCA in B cell function. Marginal zone cells generally do not express IgD and CD23 and do express IgM or IgG or IgA [39]. It is possible that they constitute a memory B cell population [15] with MT3 expression associated with memory B cells and KiB3 with unprimed B cells. UCHL-1 (CD45R0) and MB1/2H4 (CD45RA) also showed a reciprocal expression pattern in the white pulp of spleen.

2d. Peripheral Blood Mononuclear cells

Staining characteristics were analysed on a FACScan flow cytometer (Becton Dickinson). With gating based on flow scatter and size scatter, lymphocytes and monocytes were examined for their LCA expression separately. To examine the LCA phenotype of T cells, T cells were enriched by B cell depletion. Virtually all T cells were MT3 (CD45RB), PD7/26 (CD45RB) and 2B11 (CD45) positive. Two subpopulations of T cells could be clearly distinguished on the basis of CD45RA and CD45R0 reactivities (fig 8a,b). In contrast, no distinct subpopulations could be seen with KiB3 (CD45RA) staining (fig 8c). Monocytes were largely positive for 2B11 (CD45), PD7/26 and MT3 (CD45RB), and UCHL-1 (CD45R0). Despite the UCHL-1 positivity which indicates the presence of CD45R0, another anti-CD45R0 antibody, OPD4, was not reactive with peripheral monocytes (fig 9a,b), reflecting another difference in LCA glycosylation between lymphocytes and monocytes. Around 20% of the monocytes expresses CD45RA as recognized by MB1 and MT2, whereas 2H4 stains 43% of all monocytes and KiB3 staining is highly heterogeneous. Virtually all peripheral B cells were reactive with anti-CD45RA and PD7/26 (CD45RB) but not with anti-CD45R0. In addition, a significant portion of peripheral B cells were MT3 (also CD45RB) positive. Interestingly, the expression of KiB3 (another CD45RA) was again highly heterogeneous, with no distinct subpopulations distinguished. This may reflect different degrees of desialylation of peripheral blood lymphocytes, since the KiB3 epitope appears to be masked by carbohydrates in most lymphoid tissue lymphocytes.

3. Conclusion

Our findings indicate that the diversity in the LCA expression pattern is significantly greater than previously suspected. Different LCA molecules varying in peptide sequence and/or carbohydrate composition are differentially expressed in a cell-type and maturation-stage specific fashion. Although the alternate splicing of mRNA can generate at least five different LCA proteins in humans [16], the differential biosynthesis of the LCA carbohydrates, especially those on the variable regions, generates far more LCA heterogeneity. As the carbohydrate portion of the LCA molecule may well be the functionally important structure for interactions with its biological ligand(s) [39], a more precise delineation of LCA carbohydrate heterogeneity helps to understand how LCA functions. LCA embodies tyrosine phosphatase activity in lymphocytes with its well-conserved and constant cytoplasmic domain [41]; the major control of where and when to exert the enzymatic activity probably lies with the highly variable extracellular domain that provides the theoretical opportunity to interact with a variety of ligands on other cells or perhaps on the same cell. It has been shown that the LCA tyrosine phosphatase can activate the T-cell tyrosine protein kinase p56 lck that is associated with the intracellular domains of CD4 and CD8 [19]. It has also been shown that LCA is linked to CD2 as well as MHC class I on the surface of T lymphocytes [31]. Future research should focus on the identification of the ligands for the variable extracellular portions of LCA.

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TABLE 1 Diagram of LCA

DIAGRAM OF LCAs

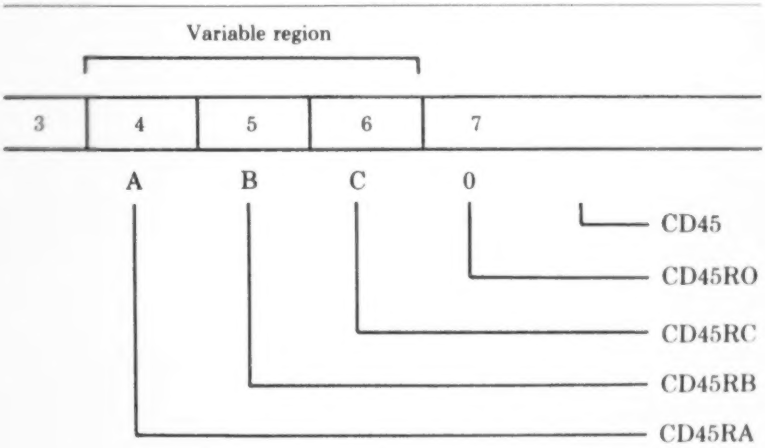
Variable region
3
4
5
6
7
A
B
C
0
CD45
CD45RO
CD45RC
CD45RB
CD45RA

Table 2. Neuraminidase Sensitivity of CD45/CD45R epitopes

# clone	CD	M.W.	N.S.	T.R.	L.A.W.	ref.
1. 2D1	CD45	220/205/190/180	-	+	+	(23)
2. 2B11	CD45	220/205/190/180	+	+	+	(43)
3. 2H4	CD45RA	220/205	-	+	+	(30,35)
4. MB1	CD45RA	220	-	+	+	(25)
5. MT2	CD45RA	220/205	+	-	+	(25)
6. KiB3	CD45RA	220/205	-	+		(13)
7. PD7/26	CD45RB	220/205/190	+	+	+	(43)
8. MT3	CD45RB	190 (220/205)	+	+		(24)
9. UCHL1	CD45R0	180	+	+	+	(33,37)
10 OPD4	CD45R0	180	+	+		(42)
11 A6	CD45R0	180	+	+	+	(2)

Note:

1. MT2 reactivity on mantle zone B cells persisted after 2 hours of neuraminidase treatment.
2. PD7/26 reactivity persisted after 15 minutes of neuraminidase treatment but completely disappeared after 30 minutes of treatment.
3. CD: cluster designation.
4. M.W.: molecular weight as detected on peripheral blood mononuclear cells.
5. T.R.: Transfectant reactivity. All antibodies were tested on LCA transfectants with and without removal of carbohydrates to establish the variable exon specificity. + indicates reactivity; - indicates no reactivity.
6. L.A.W.: Leukocyte Antigen Workshops: + indicates clustering as CD45 or CD45R in one of the workshops.

Table 3. Expression of LCA[(A)]

	<--- CD45RA	--->		<- CD45RB ->		< CD45RO >
	MB1	MT2	KiB3	PD7	MT3	UCHL-1
1.Thymocytes						
a.cortical	-	-	-	+	+	+
b.medullary	+/-	+/-	-	+	+	+/-
2.Lymph node						
a.interfollicular T	+/-	+/-	-	+	+	+/-
b.mantle B	+	+	+	+	+	-[(B)]
c.germinal center B	+	-	+	+	+[(C)]	-
d.germinal center T	-	-	-	+[(D)]	+	+
3.Spleen marginal zone B	+	+	-	+	+	-
4.Peripheral monocytes	+/-	+/-	+/-	+	+	+[(E)]
5.Peripheral T cells	+/-	+/-	+/-	+	+	+/-
6.Peripheral B cells	+	+	-/+	+	-/+	-

Note:

(A): +, majority of population is positive; -, majority of population is negative; +/-, positive and negative subpopulations.

(B): some mantle zone B cells express CD45RO at low level.

(C): weakly positive.

(D): Leu7$^+$ cells within the germinal center are PD7/26 negative (result not shown).

(E): another anti-CD45RO mab OPD4 is not reactive with peripheral monocytes.

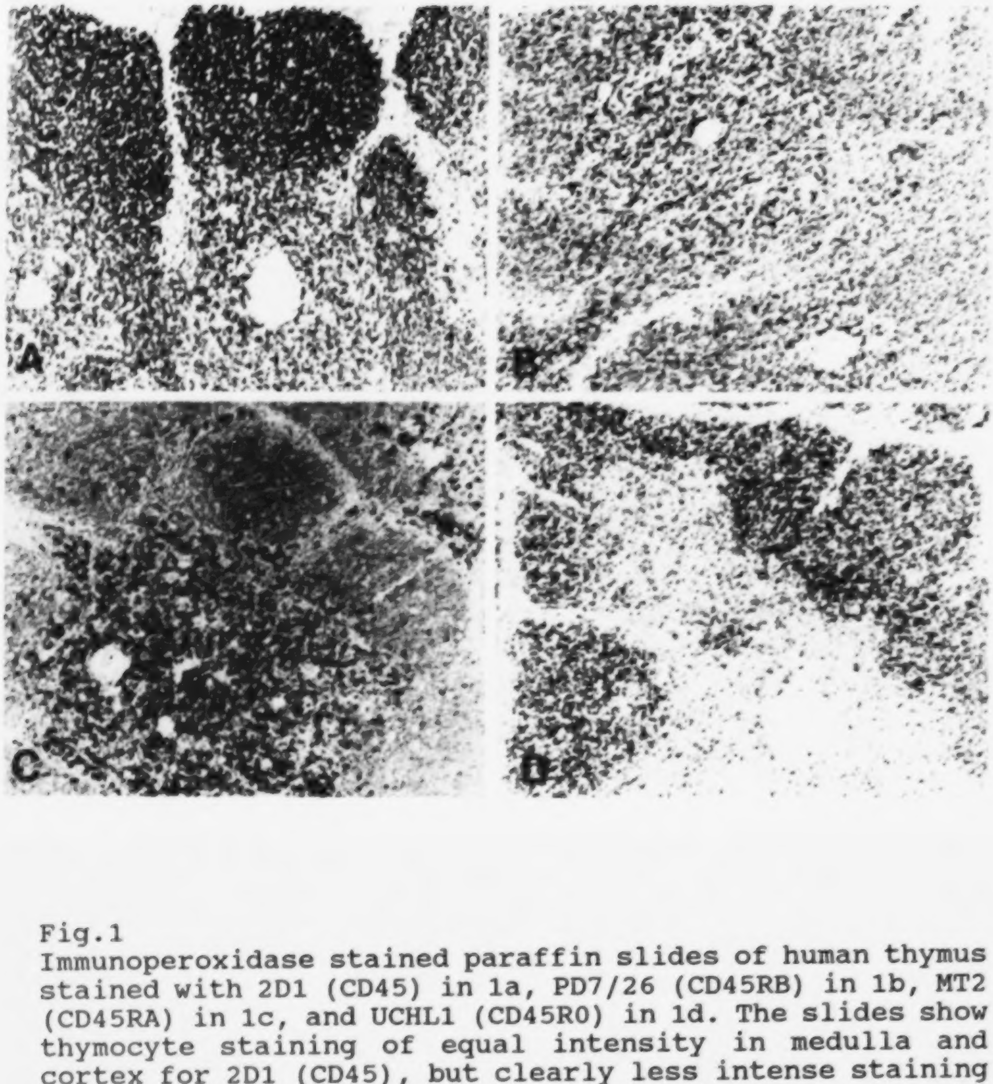

Fig.1
Immunoperoxidase stained paraffin slides of human thymus stained with 2D1 (CD45) in 1a, PD7/26 (CD45RB) in 1b, MT2 (CD45RA) in 1c, and UCHL1 (CD45R0) in 1d. The slides show thymocyte staining of equal intensity in medulla and cortex for 2D1 (CD45), but clearly less intense staining in cortex with PD7/26 (CD45RB). MT2 (CD45RA) strongly stains medulla, whereas UCHL1 (CD45R0) strongly stains cortex.

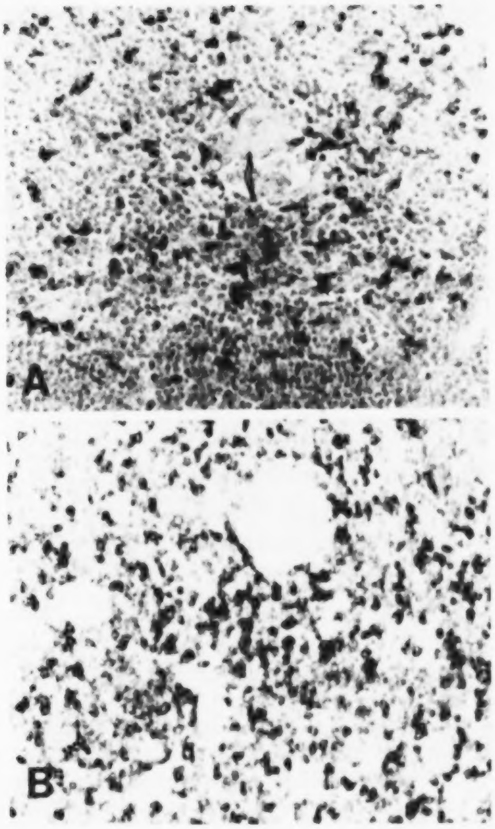

Fig.2
Immunoperoxidase stained paraffin slides of human thymus stained with MB1 (CD45RA) without (2a), and with (2b) neuraminidase pretreatment. The medulla shows an increase of MB1 positive thymocytes after neuraminidase pretreatment.

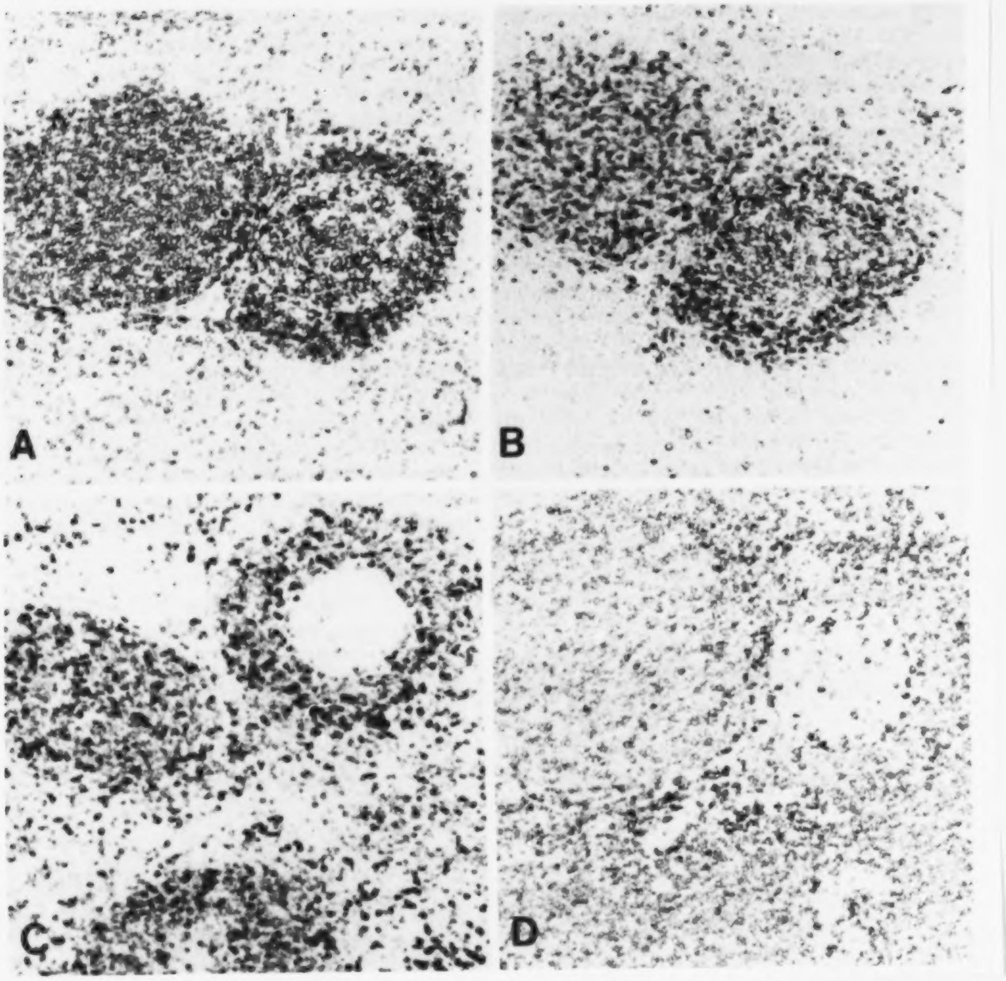

Fig.3
Immunoperoxidase stained paraffin slides of human lymph node stained with MB1 (CD45RA)(3a), KiB3 (CD45RA) (3b), MT2 (CD45RA)(3c), and 2B11 (CD45)(3d). Note positive staining of subpopulation of T cells in paracortex with MB1 and MT2 but not with KiB3. Also notice absence of staining of all germinal center cells with MT2 and very weak germinal center B cell staining with 2B11, whereas strong staining of a few small germinal center T lymphocytes can be seen with 2B11 only.

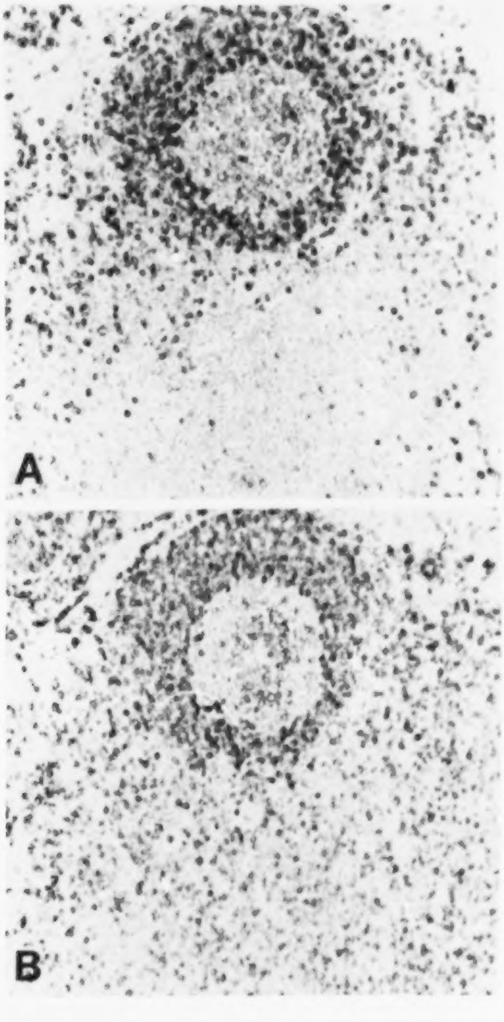

Fig.4
Immunoperoxidase stained paraffin slide of human lymph node stained with KiB3 (CD45RA) before (4a) and after removal of carbohydrates with neuraminidase and O-glycanase (4b). Note the additional positive staining of subpopulation of lymphocytes in paracortex, despite the fact that the overall intensity of the staining is the same in both slides.

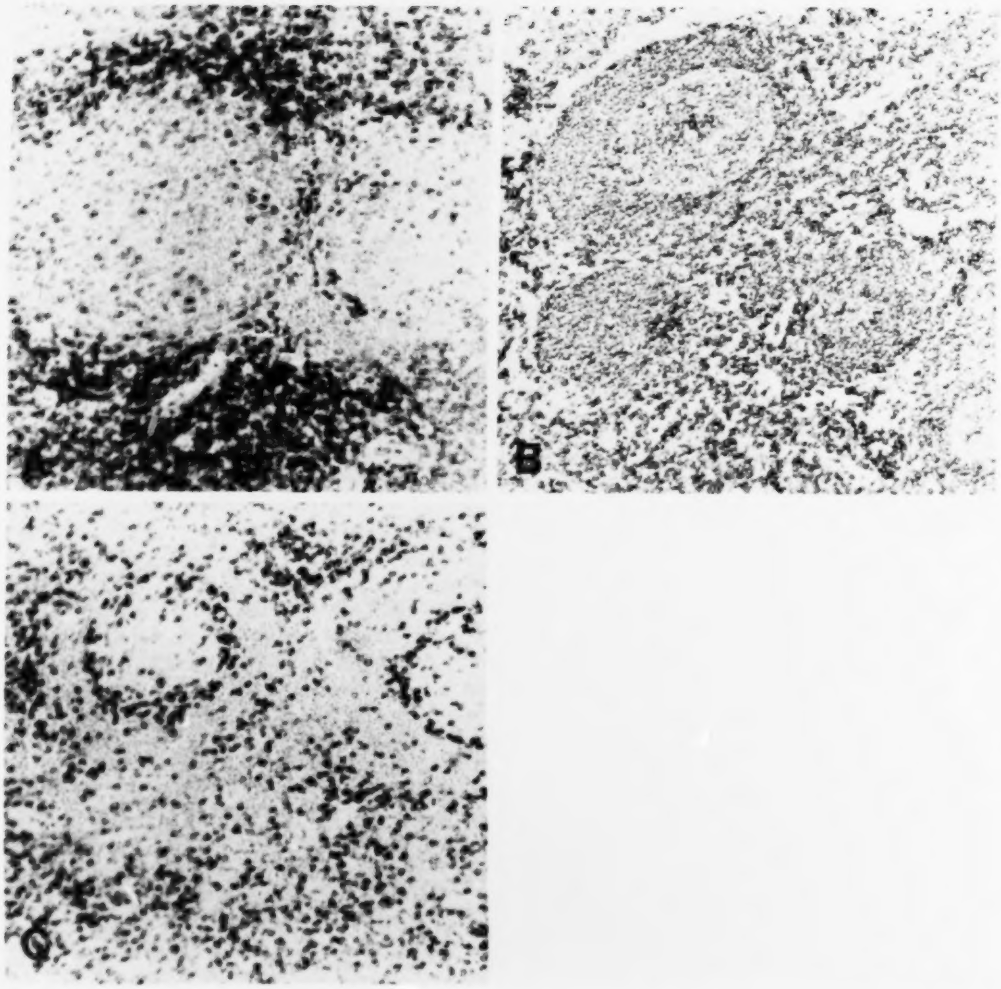

Fig.5
Immunoperoxidase stained paraffin slides of human lymph node stained with MT3 (CD45RB)(5a), PD7 (CD45RB)(5b), and UCHL1 (CD45R0)(5c). MT3 stains all T cells and a small subpopulation of B cells, whereas PD7/26 stains all cells. UCHL1 stains a large proportion of T cells in the paracortex as well as T cells in germinal centers.

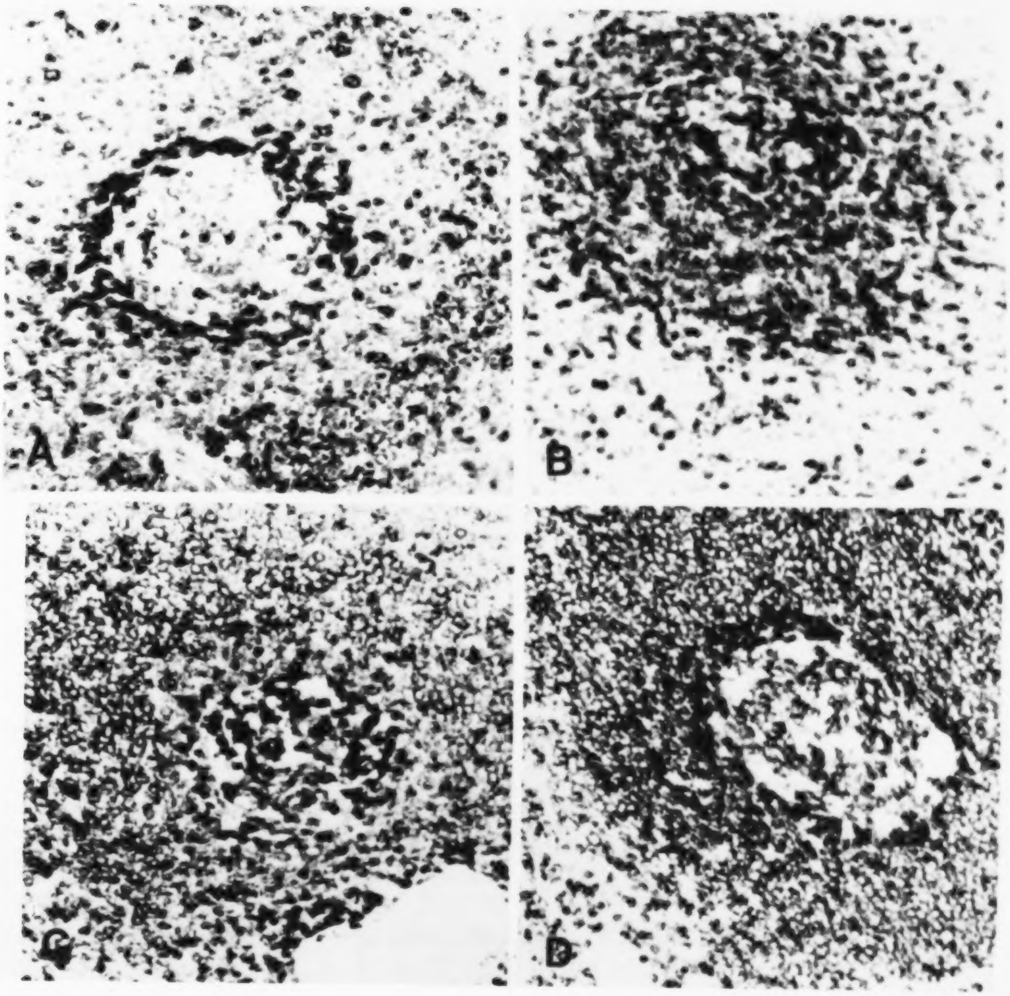

Fig. 6
Immunoperoxidase stained paraffin slides of human spleen. The figures show a follicle with (from centre to periphery) germinal center, mantle zone and marginal zone, stained with KiB3 (CD45RA) (6a), KiB3 after removal of carbohydrates with neuraminidase and O-glycanase (6b), MT3 (CD45RB)(6c), and MB1 (CD45RA) (6d). Note the additional staining of marginal zone B cells for KiB3 after removal of carbohydrates (6b). Also note the absence of mantle zone staining for MT3 in 6c, as compared to MB1 where a strongly positive staining rim of small mantle zone lymphocytes can be observed between germinal center and marginal zone (6d). The strongly positive cells in the centre of figure 6c are MT3 positive germinal centre T cells.

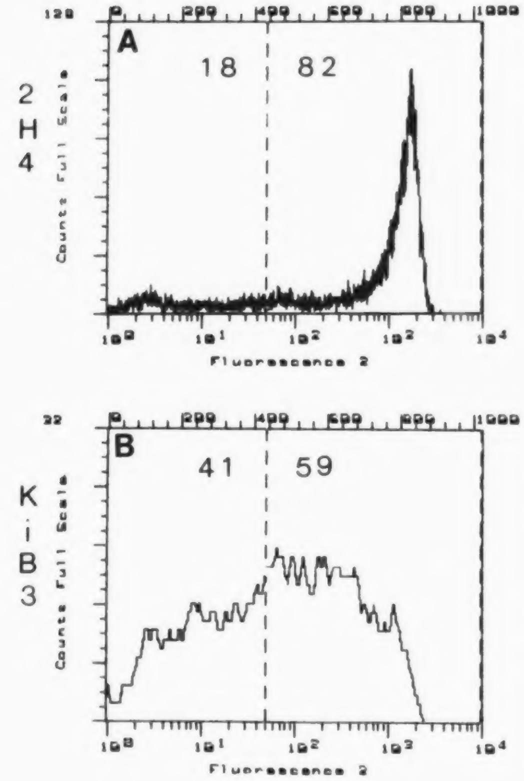

Fig.7
FACScan analysis ofperipheral blood T lymphocytes stained with 2H4 (CD45RA)(7a) and KiB3 (CD45RA)(7b). Note the heterogeneous expression of the KiB3 epitope that contrasts with the presence of a distinct 2H4 positive subpopulation.

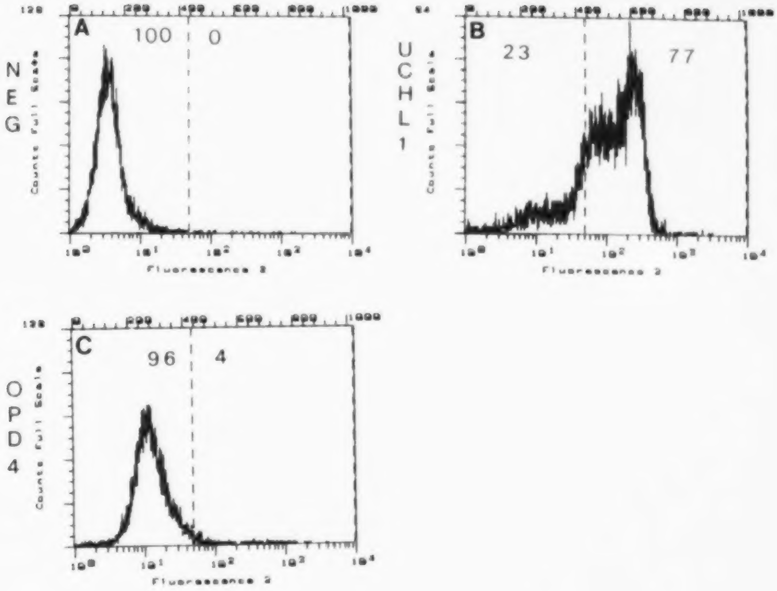

Fig. 8
FACScan analysis of peripheral blood monocytes. Negative control (8a). Note the positive staining for UCHL1 (CD45RO)(8b) versus the absence of staining with OPD4 (CD45RO)(8c), despite the fact that both recognize CD45RO epitopes.

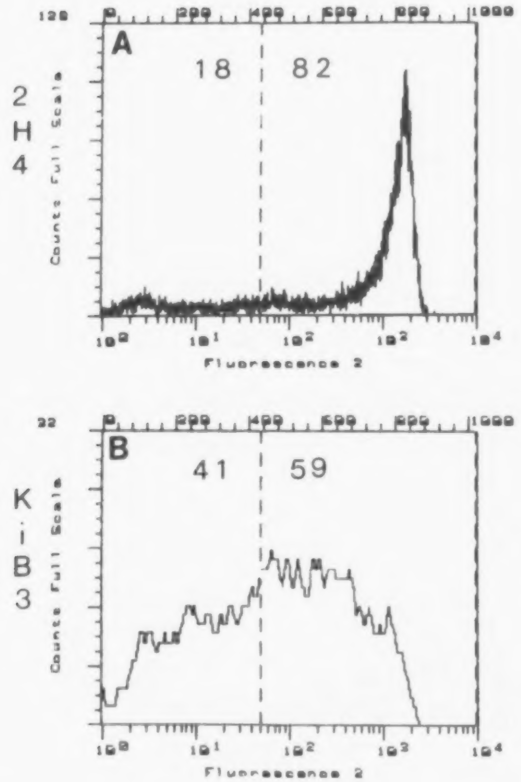

Fig.9
FACScan analysis of peripheral blood monocytes. Note that MT2 (CD45RA) stains a smaller, more distinct subpopulation (fig 9a) as compared to 2H4 (CD45RA) that has a more heterogeneous expression on monocytes.

CHAPTER 3

ANTIBODY MT3 IS REACTIVE WITH A NOVEL EXON B ASSOCIATED 190 KD CARBOHYDRATE EPITOPE OF THE LEUKOCYTE COMMON ANTIGEN COMPLEX

A version of this chapter has been published. Poppema S, Lai R, and Visser L. Journal of Immunology 1991;147:218-223.

Introduction

Leukocyte common antigen (LCA, T200, CD45) is a cell surface glycoprotein complex of approximately 200 kd, that has been found to be selectively expressed on all nucleated hemopoietic cells in man, mouse and rat [1,2,3]. LCA is composed of a family of at least five glycoproteins with apparent molecular weights of 220, 205, 190 and 180 kd, that are differentially expressed on lymphocyte subpopulations [4,5]. This heterogeneity results from the existence of several mRNA species of distinct sizes that arise by cell-type specific splicing from a common precursor gene [6,7]. It has been hypothesized that considerable additional heterogeneity results from differences in glycosylation [5]. Monoclonal antibodies have been used to define antigenic specificities present on these glycoproteins. Many antibodies react with common determinants of all four bands and are designated as anti-CD45 [8]. Several other antibodies only recognize the two higher M.W. bands of 220 kd and 205 kd as found on B cells and a subpopulation of mature T cells with suppressor-inducer activity [9], and have been designated as CD45RA. One antibody, UCHL1, is specific for the 180 kd band as present on thymocytes and a subpopulation of mature memory T cells and does not react with the population of CD45RA$^+$ suppressor-inducer cells [10]. UCHL1 is now designated as anti-CD45R0. Antibody PD7/26 reacts with nearly all hematopoietic cells, but only recognizes 220, 205 and 190 kd bands and not the 180 kd band [11]. It is now designated anti-CD45RB. Here we report antibody MT3, that predominantly reacts with a 190 kd band of the leukocyte common antigen complex and compare the tissue distribution and biochemical characteristics of the MT3 epitope to those of other leukocyte common antigen antibodies.

Materials and Methods

1. Monoclonal antibodies

Monoclonal antibodies MB1 and MT2 have been previously described [12]. MT2 is reactive with 220 and 205 kd bands and MB1 with the 220 kd band only. We also used antibody 2H4 (Coulter Clone) as a prototype CD45R antibody [9]. Monoclonal antibody UCHL1, that is reactive with a 180 kd band was kindly provided by Dr. P. Beverley [10]. Anti CD45 antibody HLe-1 was obtained from Becton and Dickinson (Mountain View, CA.). Antibody 2B11, that is reactive with all four bands of the leukocyte common antigen complex (anti-CD45), was kindly provided by Dr. D. Mason [13]. MT3 was produced in our laboratory from a fusion of X63 myeloma cells with spleen cells of a mouse immunized with Hodgkin cell line DEV that is of B cell origin [14]. Monoclonal antibody PD7/26 that is reactive with 220, 205 and 190 kd bands, but not with the 180 kd band (CD45RB), was kindly provided by Dr. D. Mason [11]. Immunohistological screening procedures on frozen lymph node tissue sections showed that supernatant of clone 27-4D1 (MT3) had a predominant reactivity with T cell areas.

2. Immunoprecipitation

Peripheral blood mononuclear cells were radioiodinated using the lactoperoxidase technique [15], and were lysed with extraction buffer containing 0.5% NP-40. After centrifugation, monoclonal antibodies were added to the supernatant and incubated for 2 hr. Subsequently, aliquots were incubated for one hour with protein A sepharose. The immunoprecipitated material was then subjected to SDS-PAGE on a 5-20% gradient gel according to the conditions of Laemmli [16] and autoradiographs were developed for 3 days.

3. Immunoblotting

Peripheral blood mononuclear cells were lysed with extraction buffer containing 0.5% NP-40. After centrifugation, the supernatant was subjected to SDS-PAGE on a 12.5 % gel according to the conditions of Laemmli [16]. The electrophoretic transfer to nitrocellulose was performed according to the conditions of Towbin [17]. The immunostainings were performed with diluted supernatants as a first step, biotinylated goat anti-mouse Ig antibodies as a second step and avidin peroxidase as a third step incubation. Peroxidase enzyme staining was done with 3-amino-9-ethylcarbazole and H_2O_2 for 10 minutes. For the preclearing experiments, peripheral blood mononuclear cell lysate was obtained as described above. The lysate was then preincubated three times for four hours with Pansorbin (S. aureus) (Calbiochem, La Jolla, Ca), coated with rabbit anti-mouse IgG Fc antibody (Jackson, Baltimore) and MT3, whereas control lysate was incubated with Pansorbin and rabbit anti mouse IgG Fc antibody only. Subsequently, the precleared lysates were subjected to the immunoblot procedures as described above, and stained with CD45 (anti HLe-1, 2B11), MB1, MT2, 2H4, PD7/26, UCHL1 and MT3.

4. Immunohistology

Five micron thick frozen tissue sections were airdried and fixed in acetone for 20 minutes. Three micron thick B5 fixed paraffin embedded tissue sections were deparaffinized through xylene and graded alcohols. The slides were incubated with 1:10 diluted supernatants for 30 minutes, followed by a second step incubation with peroxidase conjugated goat anti mouse Ig antibodies (Jackson laboratories), diluted 1:50, for 30 minutes. Primary antibodies HLe-1, 2B11, MB1, MT2, 2H4, PD7, MT3, and UCHL1 were employed. Between incubations, the sections were washed in phosphate buffered saline for 5 minutes. Peroxidase activity was visualized with 3-amino-9-ethylcarbazole and H2O2 for 15 minutes on the frozen sections and with diaminobenzidin and H2O2 for 10 minutes on the paraffin sections. Nuclear counterstaining was obtained with Mayer's Hemalum.

5. Reactivity with transfectants expressing human LCA molecules

The five LCA transfectant cell lines and the control murine pre-B lymphocyte cell line were made available to us by Dr Michel Streuli. The establishment and characteristics of these lines have been described by Streuli et al [18]. The lines were grown in RPMI medium with 10% FCS, 2mM L-glutamine and 0.5 mg/ml Geneticin. The reactivity of all antibodies was tested by two methods. First, cytospins were prepared from the cell lines. These were airdried, fixed in acetone for 10 minutes and stained with an immunoperoxidase method as described above. Secondly, the cell lines were incubated with the anti LCA antibodies and a second step fluorescein conjugated goat anti mouse antibody and flow cytometric analysis of the fluorescence was performed on a FACScan flow cytometer according to the method described by Streuli et al [18].

Results

1. Cell distribution

Immunofluorescence staining of PBL with MT3 and a second-step fluoresceinated goat anti-mouse antiserum led to staining of approximately 90% of the cells. The positive population showed a bimodal peak with approximately 40% with dull staining and 60% with bright staining. In addition, the antibody showed weak staining with monocytes (fig.1) and no reactivity with granulocytes (not shown). In bone marrow, only a small number of lymphocytes were reactive (fig. 2). On frozen and paraffin tissue sections of thymus, MT3 was found to react with the vast majority of cortical and medullary thymocytes, with relatively strong staining of a subpopulation of the medullary T cells, similar to the staining for PD7 (fig. 3). In reactive lymph nodes and tonsils, the majority of lymphocytes in T cell areas were stained, with subpopulations of strongly and weakly positive cells, similar to PD7. B cell areas were generally negative, except for a minority of around 10 to 30% of mantle zone B cells that were positive. This B cell staining was confirmed by a double-staining procedure employing fluoresceinated MT3 and phyroerythrin-labelled anti-CD19 (anti-Leu-12). This clearly differed from PD7 that stained all mantle zone B cells. T cells in germinal centers were strongly MT3 positive (fig. 4). This T cell staining was confirmed by double-staining procedure with fluorescinated MT3 and phyroerythrin-conjugated anti-CD3 (anti-Leu-4). In spleen, T cell areas and follicles stained similar to those in lymph nodes, but, in addition, staining of the majority of marginal zone cells could be observed (fig 5). The B cell staining was also confirmed by a double-staining procedure with fluorescinated MT3 and phyroerythrin-conjugated anti-CD19 (anti-Leu-12). Plasma cells in reactive lymph nodes were not reactive with MT3. Langerhans' cells in the epidermis and sinus histiocytes and epithelioid histiocytes in lymph nodes did not react with MT3 and also not with MB1, MT2, and PD7/26, whereas these cell types do react with conventional CD45 (220/205/190/180) antibodies and with UCHL1. No reactivity of MT3 was found with any non-hematopoietic cells on a panel of normal tissues. The MT3 as well as the PD7/26 reactivity could be completely abolished by the pretreatment of cells and tissue sections with neuraminidase. Staining for 2H4, a CD45RA antibody, was not influenced by neuraminidase pretreatment.

2. Biochemical characterization

In immunoprecipitation and in immunoblotting procedures MT3 was found to be reactive with a specific band with an apparent molecular weight of 190 kd, different from the 220/205 bands recognized by anti-CD45RA antibodies and the 180 kd band recognized by UCHL1 (anti-CD45RO). In addition, weak bands could be observed at the 220 and 205 kd positions. PD7/26 reacted with a 190 kd band as well, but also with 220 and 205 kd bands with about equal intensity (fig. 6). Frequently, the 220 and 205 kd bands of MT3 remained below the level of detection in immunoprecipitation as well as in immunoblot experiments. Pretreatment of the blots with neuraminidase resulted in loss of staining for MT3 as well as PD7/26 (fig. 7).

Preclearing of the lysate with MT3 lead to a loss of the 190 kd band when the precleared lysate was stained with MT3, but only to a partial loss of the 190, a complete loss of 205 and no detectable loss of the 220 kd bands when stained with PD7/26 (fig. 8).

3. Transfectants

The reactivity with the transfectants is summarised in table 1. MT3 only reacted with clones expressing the fl-LCA.338 (AB) and fl-LCA.623 (B) in contrast to PD7/26 that also reacted with fl-LCA.6 (ABC) and fl-LCA.260 (BC). The reactivity of the other antibodies was as expected, except for MT2 that did not react with these transfectants.

Discussion

Antibody MT3 is predominantly reactive with a 190 kd glycoprotein present on human lymphocytes, as shown by immunoprecipitation and by immunoblotting experiments. The antigenic determinant recognized by MT3 is sialic acid-dependent inasmuch as the staining of cells in suspensions and in fixed tissue sections as well as the staining of specific bands in immunoblots can be completely abolished by preincubation with neuraminidase. In this respect, MT3 is similar to a number of anti-LCA antibodies, including 2B11, MT2, PD7, and UCHL-1. Other reagents, like HLe-1, 2H4, and MB1 are not neuraminidase-sensitive. All forms of LCA are heavily glycosylated [20]. The neuraminidase-sensitive epitopes are endoglycosidase resistant, indicating that the critical sialic acid residues are located on O- rather than N-glycosidally linked carbohydrate chains [21].

The antigenic determinant recognized by MT3 was suspected to belong to the LCA family based on the molecular mass of approximately 200 kd and because of the results of experiments that showed a specific reduction of the 190 and 205 kd bands after preclearing of the cell membrane lysate with MT3 [22].

The results of the staining of transfectants indicate that MT3 recognizes a determinant associated with the expression exon B encoded sequences alone (B) or exon A plus exon B sequences (AB). The presence of exon C sequences as in BC and ABC appears to prevent expression of the MT3 determinant.

Antibody PD7/26 also recognizes exon B-encoded sequences and is the prototype anti-CD45RB reagent [18]. Therefore the key comparison of MT3 relates to PD7/26. A major difference is the finding that PD7/26 reacts with all four transfectants expressing exon B-encoded sequences, ie. ABC, AB, BC, and B only. PD7/26 reacts with 220, 205, and 190 kd bands on PBMC. MT3 reacts with a predominant 190 kd band and weak 220 and 205 kd bands. These findings correlate with the fact that MT3 does not react with the majority of 220 kd positive B lymphocytes.

Our results indicate that preclearing with MT3 leads to a considerable reduction in the staining intensity of the 190 kd band when lysates are stained with anti-CD45 antibodies that recognize all four bands of the LCA complex, whereas the 190 kd staining with MT3 is completely abolished. This is consistent with the notion that the MT3 epitope is different from the PD7 epitope and may only be expressed on a proportion of exon B-encoded LCA.

The MT3 staining pattern is quite different from all other anti-LCA antibodies. It reacts with all immature and mature T lymphocytes and a subpopulation of intermediate size B cells as present in the marginal zone of the white pulp of human spleen. It is not reactive with the vast majority of small B lymphocytes as found in peripheral blood, in primary follicles and the mantle zones of secondary follicles. Both PD7/26 and MT3 are weakly reactive with monocytes, and not with Langerhans cells of the epidermis, sinus histiocytes, macrophages and epithelioid histiocytes, that all are reactive with conventional anti-CD45 antibodies. These results indicate that the 190 kd variant of leucocyte common antigen as recognized by MT3 is mainly restricted to T lymphocytes.

The predominant reactivity of MT3 with T cells presents a similarity to UCHL-1 that recognized the 180 kd band. However, MT3 reacts with all mature T cells and thus clearly differs from UCHL-1 which only recocgnizes the memory T cell population [10]. In addition, MT3 reacts with a subpopulation of B cells that is mainly found in the marginal zone of spleen, whereas UCHL-1 is not reactive with a significant population of normal B cells.

The expression of the MT3 epitope on marginal zone B cells suggests that these cells may express a 190 kd band in addition to the 220 and 205 bands generally found on B cells. Marginal zone B cells are known to express membrane IgM only and not IgM and IgD as the majority of B cells in primary follicles and mantle zones of secondary follicles [23]. In addition, marginal zone cells do not express CD23 in contrast to the majority of mantle zone cells [24]. This suggests that MT3 reactivity of B cells may reflect a memory B cell population. In a study on the expression of LCA in non Hodgkin lymphomas, we have found that plasmacytomas that are CD45 positive in fact only express the 190 kDa variant as recognized by MT3 and PD7/26 in immunohistology and immunoblotting procedures, which is also confirmed by the exclusive staining of a 190 kDa band by the conventional CD45 antibodies in immunoblots [20].

Our findings further corroborate the concept that variants of the leucocyte common antigen are differentially expressed on lymphocyte subsets. The functional significance of this differential expression is largely unknown. LCA is a transmembrane glycoprotein that may be involved in transmembrane signal transduction, inasmuch as the cytoplasmic domain of LCA has tyrosine phosphatase activity [25]. Ligation of LCA can augment or inhibit T cell activation [26, 27]. No ligands for LCA are known. It can be speculated that LCA binds to molecules on interacting cells, such as APC, or alternatively to other molecules expressed on the surface of the same cells. In both situations, different isoforms of LCA as well as differences in glycosylation of these isoforms might influence binding to different other molecules on the cell surface, thus altering the signaling properties of the cell. As an example, it has been demonstrated in mice that LCA is stably associated with the CD3/TCR complex and CD4 in memory T cells, whereas these molecules exist as separate entities on naive cells [28]. The presence of TCR, CD4-associated tyrosine kinase activity and LCA -associated tyrosine phosphatase activity in one complex may explain why Ag signaling properties of memory T cells are so much more effiecient than those of naive T cells. It will be of interest to define which isoforms are involved in this and other functional activities. The availability of antibodies that are reactive with the different isoforms and glycosylation variants of these isoforms provides one of the tools needed for further functional studies.

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TABLE 1 REACTIVITY WITH LCA TRANSFECTANTS

		HLe1	2B11	2H4	MB1	MT2	PD7	MT3	UCHL1
LCA.1	O	+	-	-	-	-	-	-	+
LCA.260	BC	+	-	-	-	-	+	-	-
LCA.338	AB	+	+	+	+	-	+	+	-
LCA.6	ABC	+	-	+	+	-	+	-	-
LCA.623	B	+	-	-	-	-	+	+	-
pZip		-	-	-	-	-	-	-	-

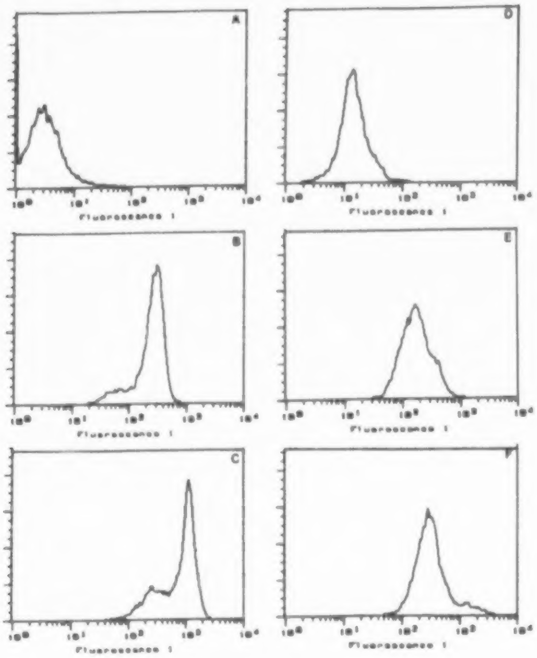

Fig 1.
Flow cytometric analysis of peripheral blood lymphocytes and monocytes with MT3 and PD7. Controls are shown in A for lymphocytes and in D for monocytes. Note the relatively strong staining of lymphocytes (B, PD7; C, MT3) compared to that of monocytes (E, PD7; F, MT3). Also note the bimodal pattern of bright- and dull-staining lymphocytes in B and C.

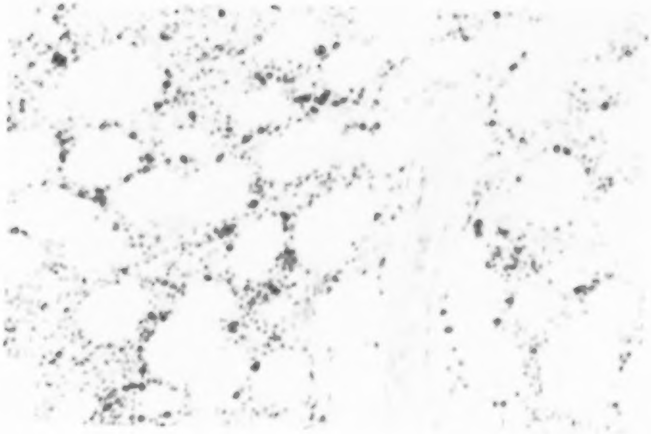

Fig.2
Immunoperoxidase stain for MT3 on bone marrow biopsy. Only small lymphocytes show positive membrane staining.

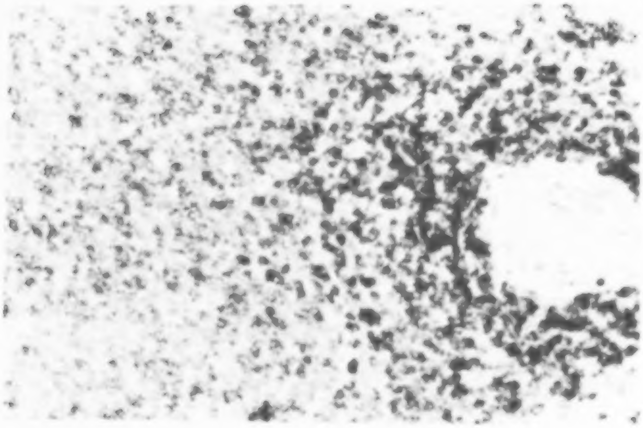

Fig.3
Immunoperoxidase staining for MT3 on paraffin tissue section of thymus. Note the strong staining of a proportion of the medullary thymocytes surrounding the Hassall's corpuscle (left) as compared to the relatively weak staining of the majority of thymocytes in the cortex (right).

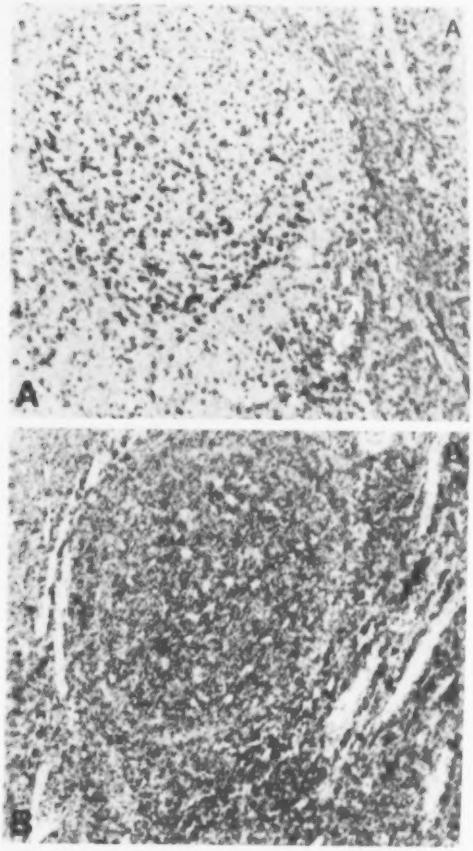

Fig.4
Immunoperoxidase staining of reactive lymph node with MT3 (A) and with PD7 (B). MT3 stains the majority of T cells in T cell area (right) and minority of T cells in germinal center of follicle (left). Mantle zone B cells and germinal center B cells are not reactive (A). PD7 stains all cells in T cell area and follicle (B).

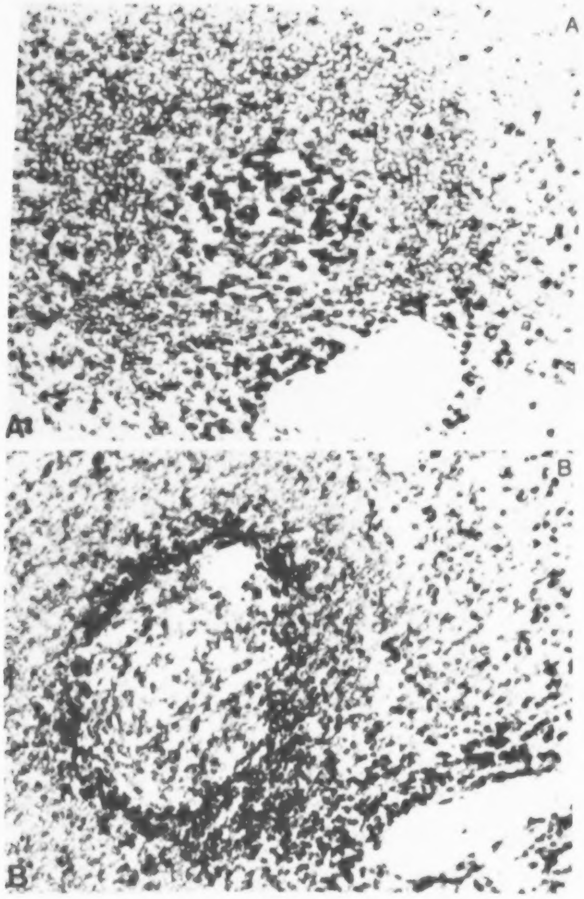

Fig.5
Immunoperoxidase staining of white pulp of spleen with MT3. Note positive staining of T cells around arteriole on bottom right and in germinal centre in the centre. The mantle zone appears as a predominantly negative rim between germinal centre and positive staining marginal zone (A). PD7/26 stains virtually all cells of the white pulp, including mantle zone cells in contrast to the absence of MT3 staining in that area.

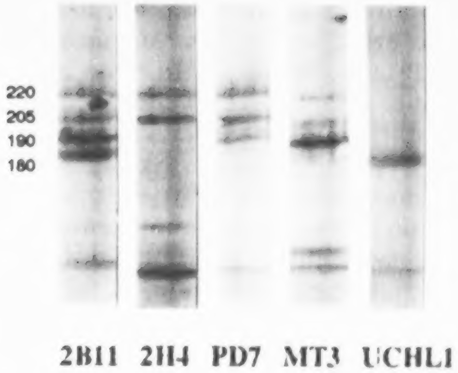

Fig.6
Immunoblots of PBMC lysate stained for 2B11 (CD45), 2H4 (CD45RA), PD7/26 and MT3, and UCHL-1 (CD45RO). MT3 shows relatively strong 190 kd band as compared to the 220 and 205 kd bands, whereas the 220-, and 205-, and 190-kd bands of PD7/26 are of equal intensity. PD7/26 and MT3 are not reactive with the 180 kd band. All reagents also show nonspecific staining of some lower molecular mass bands.

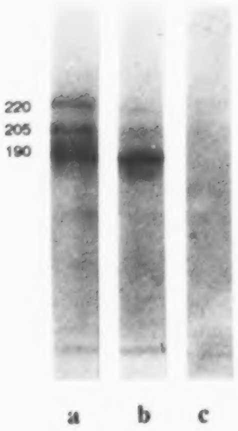

Fig.7
Additional immunoblots of PBMC stained for PD7 (lane a) and MT3 (lane b) demonstrating the predominant 190 kd staining of MT3. Pretreatment of the blot with neuraminidase results in loss of MT3 staining (lane c).

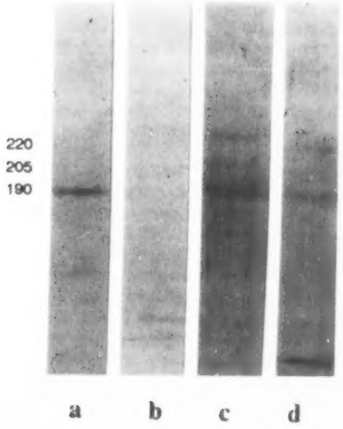

Fig.8
Imunoblots of PBMC lysate stained with MT3 (lane a), and with MT3 after preclearing with MT3 (lane b), as well as with PD7/26 (lane c) and with PD7/26 after preclearing with MT3 (lane d). Preclearing results in a complete deletion of the MT3 staining and a partial reduction of the PD7 staining, suggesting that MT3 reacts with only a proportion of exon B-encoded LCA.

CHAPTER 4

NEW ANTI CD45RB ANTIBODIES REACTIVE WITH LYMPHOCYTE SUBSETS

A version of this chapter has been submitted for publication.

Introduction

LCA (T200, CD45) is a cell surface glycoprotein complex of approximately 200 kd that is selectively expressed on all nucleated hemopoietic cells in humans [20]. It is known that differences in the peptide backbone between LCA variants are due to selective expression of three variable regions, commonly labelled A, B and C [6,19]. In the IVth Workshop on Leukocyte Antigens, only one monoclonal antibody (Mab), PD7/26 [12, 13, 20], was found to have reactivity with the B region and was assigned to the CD45RB cluster [16]. This antibody is reactive with LCA isoforms with molecular weights of 220, 205 and 190 kd, and reacts with LCA transfectants with the expression of the B variable region but not those without [19]. At this workshop we presented a new anti-LCA reagent, MT3, that had predominant reactivity with a 190 kDa band and additional weaker reactivity with 220 and 205 bands. Since then we have determined that MT3 reacts with LCA transfectants expressing exon B associated epitopes, such as B only and AB, but not BC or ABC [11]. The PD7/26 as well as the MT3 epitopes are sialic acid dependent, since both of them are sensitive to neuraminidase treatment [5, 11, 13]. Significant differences in reactivity patterns with lymphoid tissues can be shown between these two reagents: the PD7/26 epitope is present on virtually all T and B lymphocytes [5, 13], but the MT3 epitope is largely restricted to T cells and a subpopulation of B cells present in the marginal zone of spleen [5, 11]. Here we report two Mab's 6G3 and 6B6, which recognize CD45RB epitopes with characteristics different from those recognized by PD7/26 and MT3. We analysed the nature of these two epitopes using LCA transfectants and immunoblotting method. We also compared the reactivities of these two Mab's with both PD7/26 and MT3 with cells in different lymphoid compartments. The availability of these reagents provides more insight into the complexity of LCA expression in the immune system.

Materials and Methods

1. Monoclonal Antibodies and LCA Transfectants

Monoclonal antibodies 6B6 (IgG1) and 6G3 (IgG1) were produced by the fusion of myeloma cell line SP2/0 and spleen cells from a mouse immunized with human large cell non-Hodgkin lymphoma cell line VER. Antibody MT3 was previously produced in our laboratory, and PD7/26 [22] was kindly provided by Dr. D. Mason (Oxford, UK). UCHL-1 (IgG2a), an anti-CD45RO Mab, was kindly provided by Dr. P. Beverly (UK). The LCA transfectant cell lines [19] were kindly provided by Dr. M. Streuli (Boston, USA).

2. Cells

Peripheral blood was obtained from healthy donors. Mononuclear cells were isolated using Ficoll-paque (Pharmacia) following the protocol supplied by the manufacturer. Tonsils used in immunohistochemistry studies were received from the routine tonsillectomy service in a local hospital. Spleen tissue was obtained from the spleenectomy procedure for tramatized patients. Reactive lymph nodes were from routine biopsy in a local hospital.

3. Immunofluorescence staining and flow cytometry

For single stainings, peripheral mononuclear cells or LCA transfectant cells were incubated with anti-CD45 mab's for 30 minutes. After three washings with PBS (pH 7.5), FITC-conjugated goat anti-mouse antibody was added to the cell suspension for 30 minutes. For analysis of peripheral B cells, cells was first treated as above and then incubated with mouse serum (Sigma) followed by anti-CD20 conjugated with PE (Becton Dickinson). For analysis of peripheral T cells, we carried out triple stainings: after the stainings with anti-CD45RB's and PercP-conjugated anti-CD3 (Becton Dickinson) in the same protocol as for double stainings described above, UCHL-1 (IgG2a) was added to the cell suspension pre-blocked by mouse serum. The stainings were finalized with PE conjugated anti-mouse IgG2a. Flow cytometry analysis was performed with a FACScan (Becton Dickinson).

4. Lysate Preparation

Fresh peripheral mononuclear cells were treated with a lysis buffer containing 1% NP-40 on ice for 45 minutes. The supernatants were collected and centrifuged at 3000 rmp to eliminate the debris. The lysate containing supernatant was kept frozen at -20 C before use.

5. SDS-PAGE electrophoresis and immunoblotting

Lysate were treated with sample buffer containing 10% SDS, Tris-HCl (0.5M, pH 6.8) and warmed at 60 C for 15 minutes. Subsequently, they were subjected to SDS-electrophoresis (5% gel) and immunoblotted onto nitrocellulose paper. For immunodetection, nitrocellulose paper strips in an incubation buffer containing 20 mM Tris, 500 mM NaCl and Tween 20 (500 uL/L of buffer, Sigma) were first incubated with the various anti-LCA antibodies, followed by biotinylated rabbit anti-mouse mab (Dakopatts, Denmark) and peroxidase conjugated avidin (Dakopatts, Denmark). Peroxidase enzyme staining was done with 3-amino-9-ethylcarbazole (Sigma) and H2O2. Each step had a 60 minute incubation and 3 washes with incubation buffer between steps.

6. Immunohistology and neuraminidase treatment

Three-micron thick B5-fixed paraffin-embedded tissue sections were deparaffinized through xylene and graded alcohols. The slide were incubated with 1:10 diluted supernatants for 30 minutes, followed by a second step incubation with peroxidase-conjugated rabbit anti-mouse Ig antibodies (Jackson Lab.), diluted 1:50, for 30 minutes. Peroxidase activity was visualized with 3-amino-9-ethylcarbazole and H2O2 for 10 minutes. For neuraminidase treatment, immunoblots were incubated with 10 ug/mL of neuraminidase in PBS (pH 7.5) for 15 minutes at 37 C. The subsequent steps in immunodetection were the same as above.

Results

1. Biochemical Analysis of the epitopes recognized by the anti-CD45RB antibodies

Reactivities of the different Mab's (6G3, 6B6, MT3 and PD7/26) with LCA transfectants were analysed by flow cytometry. 6G3 and 6B6 were strongly reactive with all four clones carrying exon B encoded sequences (figure 1). The results strongly suggest that 6B6 and 6G3 are anti-CD45RB antibodies.

The identities of 6B6 and 6G3 as anti-CD45RB reagents were further confirmed by the molecular weight studies utilizing immunoblotting technique (figure 2). Using lysates prepared from peripheral blood mononuclear cells, we observed that PD7/26 recognized three bands of equal intensity at 220, 205 and 190 kd. 6G3 as well as 6B6 had similar patterns as PD7/26. MT3 had a strong 190 kd band and two weak bands at 220 and 205 kd. We also tested whether the epitopes recognized by different anti-CD45RB mab's were carbohydrate associated structures. Immunoblots prepared from peripheral blood mononuclear cells stripped of sialic acid by neuraminidase treatment were stained with anti-CD45RB Mab's. PD7/26, MT3 and 6G3 were not reactive with the sialic acid-deprived LCA, whereas 6B6 as well as the anti-CD45 marker HLe-1 remained reactive with the LCA complex without sialic acid. Further removal of either O-linked or N-linked carbohydrates did not abrogate the reactivity of these two Mab's (results not shown). These results indicate that the epitopes recognized by PD7/26, MT3 and 6G3 are carbohydrate associated structures whereas the 6B6 epitope is likely to be a protein structure.

2. Reactivity patterns in lymphoid tissue

All four anti-CD45RB Mab's showed stronger reactivity with medullary thymocytes than with cortical thymocytes. However, within the medullary thymocytes population, there were two distinct subpopulations - 6G3 strong and 6G3 weak. This difference of expression intensity shown with routine immunohistochemistry was also evident with MT3 staining, but not with 6B6 or PD7/26. For the tonsils (fig. 3), the reactivity of 6B6 was similar to PD7/26: both antibodies reacted with almost all cells at equal intensity. MT3 had a more restricted reactivity pattern, being strongly reactive with all T cells, but also with up to 30 % of mantle zone B cells and very weakly with the germinal center B cells. Staining with B cells was weaker than with T cells. 6G3 was largely not reactive with the germinal center B cells but it stained lymphocyte subpopulations within the mantle zone with variable intensity. The reactivities of the anti-CD45RB's with reactive lymph nodes were similiar to those found in tonsils.

Within the spleen, the reactivity of all anti-CD45RB mab's toward the mantle zone and germinal center followed the same pattern as in tonsils and lymph nodes. Marginal zone B cells were recognized by all anti-CD45RB Mab's, including MT3.

3. Reactivity with peripheral blood lymphocyte subsets

With a double staining procedure of peripheral blood mononuclear cells, cells gated for CD20 positivity could be analysed for their CD45RB expression and coexpression of IgM, IgD and CD23. PD7/26 and 6B6 were strongly reactive with most of the peripheral B cells. In contrast, these B cells were largely 6G3/MT3^{dim} with a distinct smaller population being 6G3/MT3^{bright} (figure 4). The 6G3/MT3^{dim} population coexpressed IgM and IgD as well as CD23, whereas the 6G3/MT3^{bright} population expressed IgM, but not IgD and CD23 (data not shown).

Peripheral T cells gated for CD3 positivity were also analysed by flow cytometry for their expression of CD45RB. Although staining with PD7/26 and 6B6 did show the existence of CD45RB^{bright} and CD45RB^{dim} subpopulations, the CD45RB^{dim} subpopulation was much more evident with MT3 and 6G3 staining (figure 5). We further studied the relationships between the CD45RB dim/bright peripheral T cells and memory-T cell marker UCHL-1 (anti-CD45RO). Double stainings of peripheral T cells with UCHL-1 and the panel of anti-CD45RB's were performed on 5 healthy individuals of different ages. All four anti-CD45RB Mab's showed the presence of at least three populations of CD3+ cells: 1) CD45RO^{bright}/CD45RB^{dim}; 2) CD45RO^{bright}/CD45RB^{bright}; and 3) CD45RO^{dim}/CD45RB^{bright}. In addition, MT3 and 6G3 staining showed a fourth population which was CD45RO^{dim}/CD45RB^{dim} (figure 6). This small population was more prominent in the blood from the older donors. Furthermore, we observed that the CD45RB^{dim} cells were largely found in the CD4+ T cell subset but not in CD8^{+} subsets. The double stain for CD45RB and CD56 showed that most CD56^{+} cells were CD45RB^{bright} (figure 7).

Discussion

Based on their reactivities with the LCA transfectants and on the molecular weight studies, we conclude that 6B6 and 6G3 are anti-CD45RB Mab's. These two new mab's share the basic common reactivity pattern with PD7/26, the prototypic anti-CD45RB, which is reactive with virtually all lymphocytes found in various lymphoid compartments. However, they differ from PD7/26 in some aspects: (a) the 6B6 epitope is most likely expressed on the protein backbone of LCA while the other anti-CD45RB mab's recognize neuraminidase sensitive epitopes; (b) 6G3 is not reactive with the germinal center B cells and shows pronouced differences in staining intensity on T and B cells. With a double immunofluorescence staining technique analysed by FACSCAN, we found that the staining intensity of 6G3 is largely in direct proportion to that of MT3 (not shown). This suggests that 6G3^{dim} cells corresponds to the MT3 negative cells with our immunoperoxiase stainings on tissuer sections. Similarly, 6G3^{bright} cells corresponds to MT3 positive cells. Therefore, 6G3 and MT3 recognize different but closely related epitopes of CD45RB. As both the 6G3 and MT3 epitopes are sialic acid associated and their reactivity patterns are restrictive, it can be concluded that the LCA B region encoded peptides are differentially glycosylated on different cell types. Similar conclusion can also be drawn based on the fact that germinal center B cells are positive for 6B6 and PD7/26 but largely negative for MT3 and 6G3. This phenomenon probably relates to the hypoglycosylated state of germinal center B cells and the corresponding expression of the peanut agglutinin receptor in the germinal center [17].

With MT3 and 6G3, we were able to distinguish at least two B subsets, namely MT3 negative (or 6G3^{dim}) and MT3 positive (or 6G3^{bright}) in various lymphoid compartments such as mantle zones and the peripheral blood. They may represent cells at different stages of differentiation. As marginal zone B cells were postulated as memory B cells [11], MT3^{+}/6G3^{bright} B cells may have similar functional characteristics. This is supported by the findings that the 6G3^{dim}/MT3^{-} B cells coexpressed IgM, IgD and CD23 suggesting that this is a virginal B cell population, whereas the 6G3^{bright}/MT3^{+} B cells only expressed IgM, and they were both IgD and CD23 negative.

We also compared reactivities of various anti-CD45RB's with peripheral T cells. The CD45RB^{dim} CD3 positive subsets are more prominent with 6G3 or MT3 than with PD7/27 or 6B6. This suggests that a portion of peripheral T cells is 6B6 (or PD7/26) bright and 6G3 (or MT3) dim. In fact, the CD3^{+}/CD45RB^{dim}/CD45RO^{bright} subset which is predominantly CD4^{+} is identifiable with 6G3 or MT3 staining. This presumably reflects the fact that this subset of T cells (mostly CD4^{+}) is selectively hyposialylated on the CD45RB region such that MT3 and 6G3 epitopes are downregulated. The CD45RB expression of T cells has been mostly studied in the mouse model [1,2,6]. The concept is that the CD45RB^{bright} T cells represents the virginal TH1 cells whereas the CD45RB^{dim} T cells contain both memory TH1 and TH2 cells [7]. The functional delineation of T cells on the basis of CD45RB in humans is much less clear, although attempts to delineate human functional subsets on the basis of cytokine production have been made [3,8]. In this study, we demonstrated the distinction of at least four different T subsets: 1) CD45RB^{dim}/CD45RO^{bright}; 2) CD45RB^{bright}/CD45RO^{bright}; 3) CD45RB^{bright}/CD45RO^{dim}; and 4) CD45RB^{dim}/CD45RO^{dim}. How these different T subsets fit into the TH1/TH2 model remains to be studied, and the new anti-CD45RB's promise to provide useful tools in this regard. This heterogeneity within the pattern of CD45RO expression has also been observed by others [4,23]. The CD45RO^{dim} T cells may represent 'transitional T cells' [23], and are mostly CD45RB^{bright}.

In summary, we produced and analysed two new anti-CD45RB Mab's and compared them with PD7/26 and MT3. The differences in reactivities among these Mab's provide insight into the role of glycosylation of the CD45RB region in creating the observed complexity of LCA expression within the immune system. Similar differential expression of CD45 glycosylation has also been observed in the CD45RA region [5]. The functional significance of these unique glycosylations is unknown. Since LCA as a tyrosine phosphatase is capable of interacting with many different cell surface molecules [9,15,18,21], it is conceivable that heterogeneity in glycosylation on top of the rather limited variation of the peptide backbone plays a key role in determining the exact ligands. The identification of functionally different subsets in both T and B cells also requires further study.

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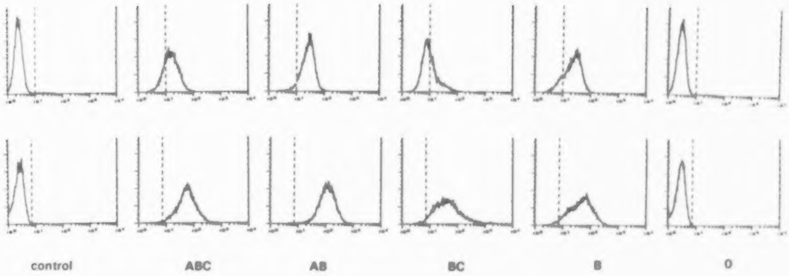


Fig.1
FACScan analysis of LCA transfectants with 6B6 (upper panel) and 6G3 (lower panel). Both antibodies show positive staining with the transfectants expressing ABC, AB, BC and B, whereas the control cells and the transfectant expressing 0 only are negative. This indicates that both antibodies are reactive with an epitope associated with the B exon of LCA.

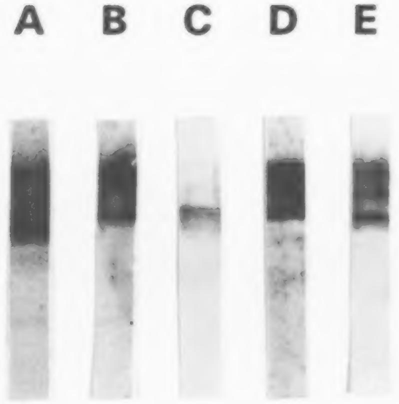


Fig.2
Western blots of lysates prepared from peripheral blood lymphocytes stained with HLe-1 (lane A) as a CD45 control, showing 4 bands at 220, 205, 190 and 180 kd, 6B6 (lane B), MT3 (lane C), PD7/26 (lane D) and 6G3 (lane E). It can be seen that 6B6, PD7/26 and 6G3 stain three bands of 220, 205 and 190 kd with approximately equal intensity, whereas MT3 shows prominent staining of the 190 kDa band and extremely weak staining of the 220 and 205 bands.

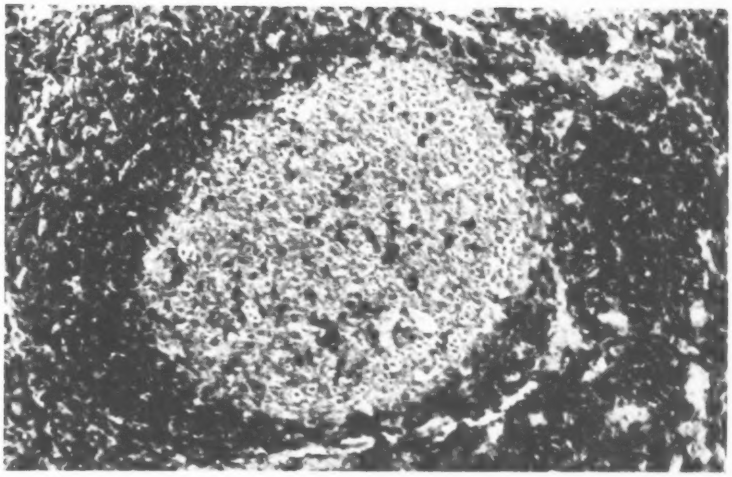


Fig.3a
Immunoalkaline phosphatase staining for 6G3 on the reactive follicle in tonsils. The germinal center B cells are negative, mantle zone B cells show variable staining with dim and bright cells, and extrafollicular T cells show dim and bright subsets.

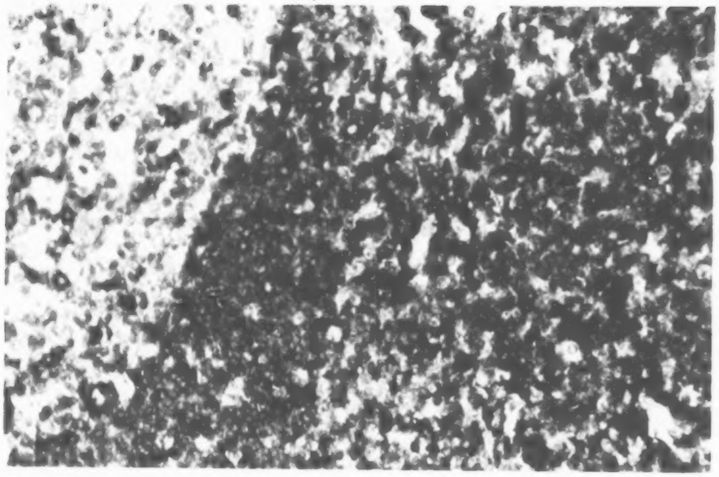


Fig.3b
Higher magnification of the same follicle stained for 6G3 shows negative germinal center (left) with few positive T cells, dim and bright staining mantle zone cells (centre) and dim and bright staining extrafollicular T cells (right).

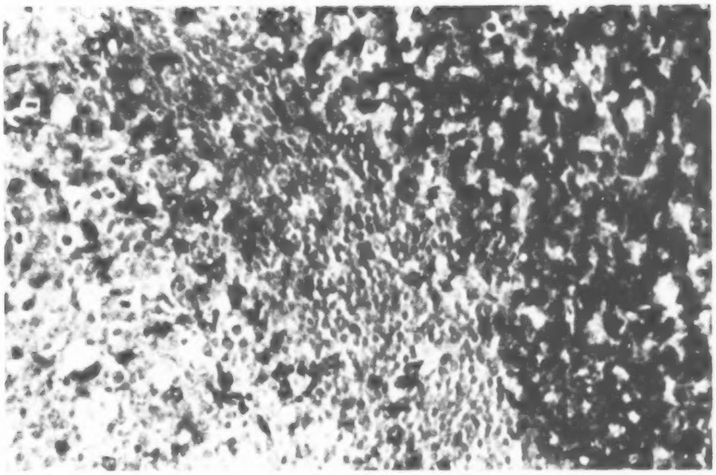
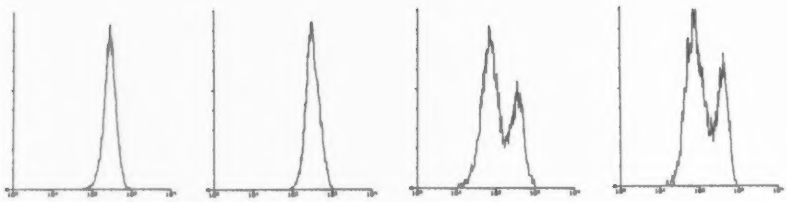
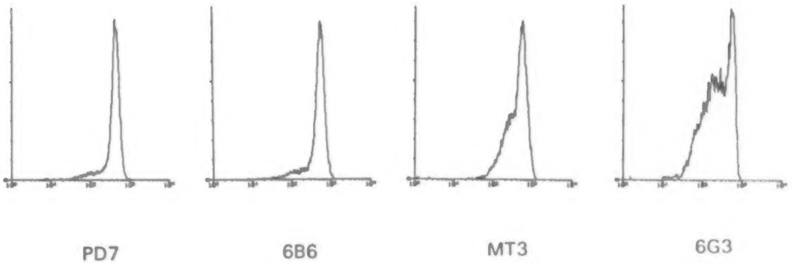


Fig.3c
High magnification of same follicle stained for MT3 shows negative germinal center (left) with few positive T cells, negative and positive mantle zone B cells (centre) and dim and bright staining extrafollicular T cells (right).



PD7 6B6 MT3 6G3

Fig.4
Two-color analysis by FACScan for CD20 and the four anti-CD45RB reagents. The results show an one-peak distribution with PD7/26 and 6B6, whereas MT3 and 6G3 identify a major dim and a minor bright population.



PD7 6B6 MT3 6G3

Fig.5
Two-color analysis by FACScan for CD3 and the four anti-CD45RB reagents. The results show minor dim populations with PD7/26 and 6B6 and major dim populations with MT3 and especially 6G3.

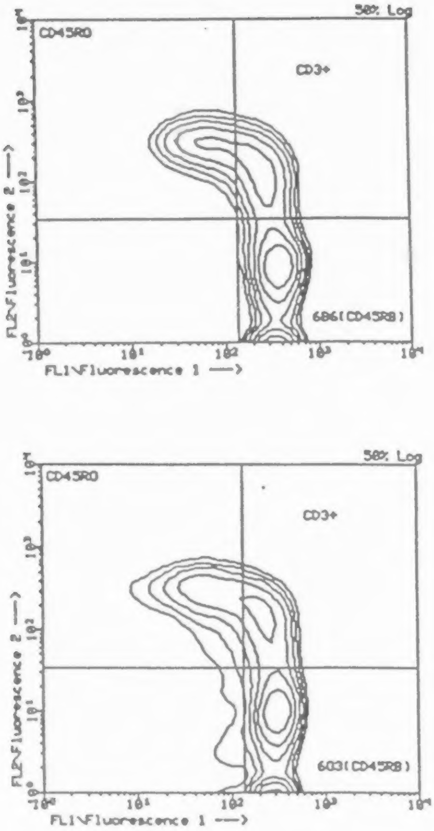


Fig.6
Three-color analysis by FACScan of CD3 positive cells for CD45RO versus 6B6 or 6G3. The results show three populations ($\mathrm{RO}^{\mathrm{bright}}/\mathrm{RB}^{\mathrm{dim}}$; $\mathrm{RO}^{\mathrm{bright}}/\mathrm{RB}^{\mathrm{bright}}$; and $\mathrm{RO}^{\mathrm{dim}}/\mathrm{RB}^{\mathrm{bright}}$) with 6B6, whereas 6G3 identifies an additional small population of $\mathrm{RO}^{\mathrm{dim}}/\mathrm{RB}^{\mathrm{dim}}$ cells.

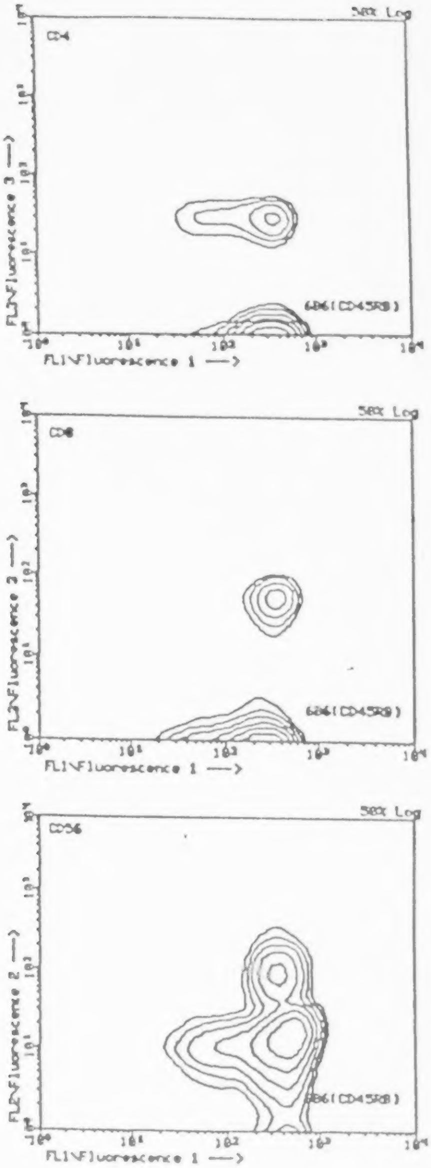


Fig.7
Two color analysis by FACScan CD4, CD8 and CD56 versus CD45RB (6B6). The results demonstrate the presence of cells with dim CD45RB expression in the CD4 subset, but not the CD8 subset, including both the CD8 bright and dim populations. In addition, a CD56^{dim} cells are uniformly CD45RB^{dim}.

CHAPTER 5

MONOCLONAL ANTIBODY OPD4 IS REACTIVE WITH CD45RO, BUT DIFFERS FROM UCHL1 BY THE ABSENCE OF MONOCYTE REACTIVITY

A version of this chapter has been published. Poppema, S, Lai, R and Visser, L. American Journal of Pathology 1991; 139:725-729.

Introduction

Monoclonal antibody OPD4 was described as recognizing a helper/inducer T cell subset on paraffin tissue sections. The molecular weight of the OPD4 antigen was reported as 200 kd. The antibody was found to be reactive with about half of the cases of T cell lymphomas. It was suggested that OPD4 may be useful for the diagnosis and study of malignant lymphomas and other related diseases [1]. Recently, OPD4 has become commercially available for such studies. The reported distribution and the molecular weight of the OPD4 antigen resemble those of the restricted leukocyte common antigen, CD45RO, as recognized by monoclonal antibody UCHL1 [2,3]. UCHL1 recognizes a restricted leukocyte common antigen isoform with a molecular weight of 180kd that is expressed on a lymphocyte subset of memory T cells [4]. Therefore, we have analysed the biochemical characteristics, the tissue distribution and the reactivity of OPD4 with LCA transfectants and compared the results with those of UCHL1.

Materials and Methods

1. Reagents

Monoclonal antibody OPD4 was a gift of Dr. T. Yoshino. Antibody UCHL1 (CD45RO) was a gift by Dr. P. Beverley. Other reagents used for comparison were HLe-1 (CD45), obtained from Becton and Dickinson, 2H4 (CD45RA) from Coulter, MB1 (CD45RA) and MT3 (CD45RB), both produced in our own lab, and PD7/26 (CD45RB), that was kindly provided by Dr. D. Mason [5,6,7,8,9]. The five LCA transfectant cell lines and the control murine pre-B lymphocyte cell line were made available to us by Dr. M. Streuli. The establishment and characteristics of these lines have been described by Streuli et al [10]. The lines were grown in RPMI medium with 10% FCS, 3mM L-glutamine and 0.5 mg/ml Geneticin. The cell lines were incubated with the anti LCA antibodies and a second step fluorescein conjugated goat anti-mouse antibody. Flow cytometric analysis of the fluorescence was performed on a FACScan flow cytometer.

2. Immunoblotting

Peripheral blood mononuclear cells were lysed with extraction buffer containing 0.5% NP-40. After centrifugation, the supernatant was subjected to SDS-PAGE on a 5% gel according to the conditions of Laemmli [11]. The electrophoretic transfer to nitrocellulose was performed according to the conditions of Towbin [12]. The immunostainings were performed with diluted supernatants as a first step, biotinylated goat anti-mouse Ig antibodies as a second step, and avidin peroxidase as a third step. Peroxidase enzyme staining was done with 3-amino-ethylcarbazole and H2O2 for 10 minutes.

3. FACScan Staining and Blocking Experiments

Peripheral blood mononuclear cells were incubated with OPD4 or control antibodies and subsequently with fluorescein-conjugated goat anti-mouse Ig antibodies (Becton Dickinson), and lymphocytes and monocytes were analyzed separately by the setting of the appropriate gates.

For the blocking experiments, the cells were first incubated with unlabeled OPD4 (mouse Ig subclass IgG1) and subsequently with UCHL1 (mouse Ig subclass IgG2), followed by a second step reagent that reacts with mouse IgG2. In addition, cells were preincubated with UCHL1, followed by OPD4 and a second step anti-mouse IgG1 reagent. The staining intensity was compared to that without preincubation with the first reagent.

4. Immunohistology

Five lymph nodes, three tonsils and three spleens submitted for diagnostic reasons and five fragments of normal thymus tissue, removed for technical reasons during cardiac surgery, were either fresh frozen in OCT or fixed in B5 or formalin. In addition, frozen tissue and B5 fixed tissue of twelve T cell lymphomas that previously had been extensively phenotyped was studied. Five micron thick frozen tissue sections were airdried and fixed in acetone for 20 minutes. Three micron thick B5 fixed paraffin embedded tissue sections were deparaffinized through xylene and graded alcohols. The slides were incubated with 1:10 diluted supernatants for 30 minutes, followed by a second step incubation with peroxidase-conjugated goat anti-mouse Ig antibodies (Jackson Laboratories), diluted 1:50, for 30 minutes. In between incubations the sections were washed in phosphate buffered saline for 5 minutes. Peroxidase activity was visualised with 3-amino-9-ethylcarbazole and H2O2 for 15 minutes on the frozen sections and with diaminobenzidin and H2O2 for 10 minutes on the paraffin sections. Nuclear counterstaining was obtained using Mayer's Hemalum. Cytospins of peripheral blood mononuclear cells were stained with the same methods as the frozen sections.

To test for sialic acid dependence of the antigenic epitopes, slides were incubated with neuraminidase (Sigma) at a concentration of 10 milliunits per mL of incubation buffer, at 37 C for 30 minutes and subsequently washed in phosphate buffer [13]. Next the staining was performed as described above. Staining for 2H4, that is not neuraminidase-sensitive, was performed as a control.

Results

1. Biochemical Characteristics

The molecular weight of the antigen recognized by OPD4 was 180 kd, very similar to that of the LCA isoform recognized by UCHL1 (fig. 1). It clearly differed from the molecular weights of other restricted LCA antigens such as those recognized by CD45RA or CD45RB reagents. The antigenic determinant was sensitive to neuraminidase, once again similar to the UCHL1 determinant.

2. Flow Cytometric Analysis

FACScan analysis of peripheral blood lymphocytes and monocytes indicated that approximately 60 percent of the peripheral blood lymphocytes stained for OPD4 with a staining profile identical to that of UCHL1 (fig. 2). However, OPD4 had no or only very weak staining with peripheral blood monocytes, whereas UCHL1 showed relatively strong monocyte staining (fig. 2). This was confirmed by immunoperoxidase staining on cytospins of peripheral blood mononuclear cells. Also, OPD4 did not react with granulocytes, whereas UCHL1 did. The blocking experiments showed that OPD4 and UCHL1 react with different epitopes, since OPD4 did not block staining with UCHL1, and UCHL1 did not block staining with OPD4.

In tissue sections, the staining pattern of OPD4 was identical to that of UCHL1. Both antibodies stained in acetone- fixed frozen sections as well as in B5-fixed and in formalin-fixed paraffin sections. However, staining intensity and tissue morphology were superior in the B5-fixed sections. OPD4 as well as UCHL1 stained cortical thymocytes and a proportion of the medullary thymocytes (fig. 3a). In addition, both reagents stained about 60% of the lymphocytes in lymph nodes, tonsils and spleen in the known T-cell areas. No staining of the majority of lymphocytes in follicles (B-cell areas) was observed (fig 3b). In a series of T cell lymphomas, including lymphoblastic, small lymphocytic, mixed small and large cell and large cell cases, 83% of the cases were positive for both UCHL1 and OPD4, with identical staining in all cases.

3. Reactivity with transfectants

OPD4 and UCHL1 were tested against a panel of LCA transfectants, expressing the ABC, AB, BC, B or none (O) of the variable exon encoded regions. A series of known CD45, CD45RA and CD45RB reagents were used as controls. OPD4 was found to only react with the transfectant expressing none of the variable exon encoded regions and was identical to UCHL1 in this respect. The staining results are illustrated in fig. 4 and summarized in table 5.

Discussion

The results of the transfectant staining clearly indicate that OPD4 reacts with a leukocyte common antigen epitope that is only exposed in the absence of expression of variable region encoded sequences. Antibodies reactive with this determinant, such as UCHL1 have been designated to cluster CD45RO [2,5]. This conclusion is supported by the results of the gel electrophoresis and immunoblots that indicate a molecular weight of 180 kd for the OPD4 antigen, again similar to that of UCHL1 [3].

The staining pattern of OPD4 indicates reactivity with a subset of T lymphocytes. The only difference between OPD4 and UCHL1 is the absence of detectable staining of monocytes and granulocytes by OPD4. This may well be the result of differences in carbohydrate composition between lymphocytes and monocytes and granulocytes that may be recognized by the two reagents that both react with neuraminidase-sensitive determinants. In our immunoblots it can be observed that UCHL1 and OPD4 recognize two bands around 180 kd. An argument for the two reagents recognizing different epitopes of the CD45RO antigen is provided by the absence of blocking in our flow cytometric analyses.

In the original description of OPD4, the reactivity in thymus was reported as being confined to few thymocytes in cortex as well as medulla [1]. In our tests the staining pattern was identical to that of UCHL1 with weak staining of the majority of cortical thymocytes and stronger staining of a minority of thymocytes. Although we cannot exclude differences in antigen expression between Japanese and North American individuals or another form of polymorphism of the antigen expression, the most likely explanation is a difference in the tissue preparation and/or staining procedures used, resulting in staining only of the strongly positive thymocyte minority in the previous study. This is strongly suggested by comparison of the staining intensity of thymocytes and T lymphocytes in our figures 3a and 3b as compared to figures 3b and 3c in the previous paper [1]. Our figures illustrate the results in B5-fixed tissue, whereas Yoshino et al used formalin-fixed tissue [1].

In conclusion, OPD4 is an anti-CD45RO reagent similar to UCHL1. The absence of monocyte and granulocyte reactivity may be due to differences in carbohydrate composition and suggests that the two reagents recognize different epitopes. It may be worthwhile to compare these antibodies in functional studies involving lymphocytes and monocytes. Since OPD4 and UCHL1 react with epitopes of the same antigen on T lymphocytes it does not appear useful to apply both reagents in the immunophenotyping of lymphomas on paraffin tissue sections.

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TABLE 1 REACTIVITY WITH LCA TRANSFECTANTS

		HLe1	2H4	MB1	PD7	MT3	UCHL1	OPD4
LCA.1	O	+	-	-	-	-	+	+
LCA.260	BC	+	-	-	+	-	-	-
LCA.338	AB	+	+	+	+	+	-	-
LCA.6	ABC	+	+	+	+	-	-	-
LCA.623	B	+	-	-	+	+	-	-
pZip		-	-	-	-	-	-	-

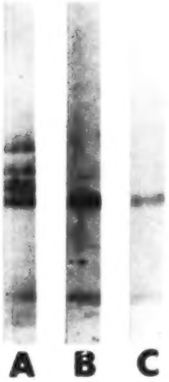


Fig.1
Immunoblots of peripheral blood lymphocytes stained for 2B11 (CD45) (a), UCHL1 (b) and OPD4 (c) showing the 4 bands at 220, 205, 190 and 180 as stained by 2B11, and identical bands at 180 kd only as stained by UCHL1 and OPD4.

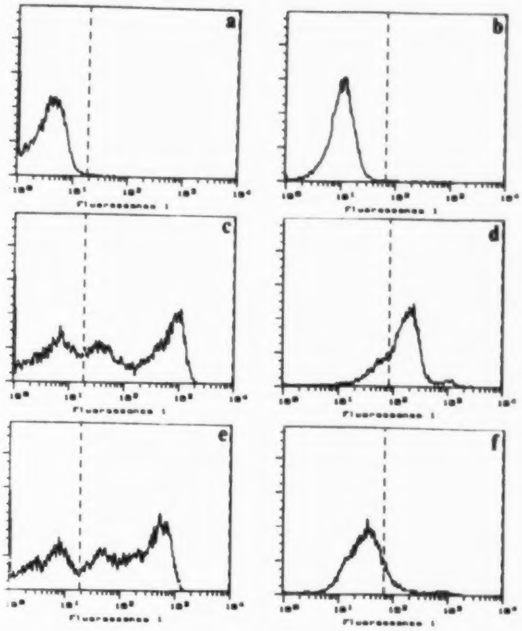
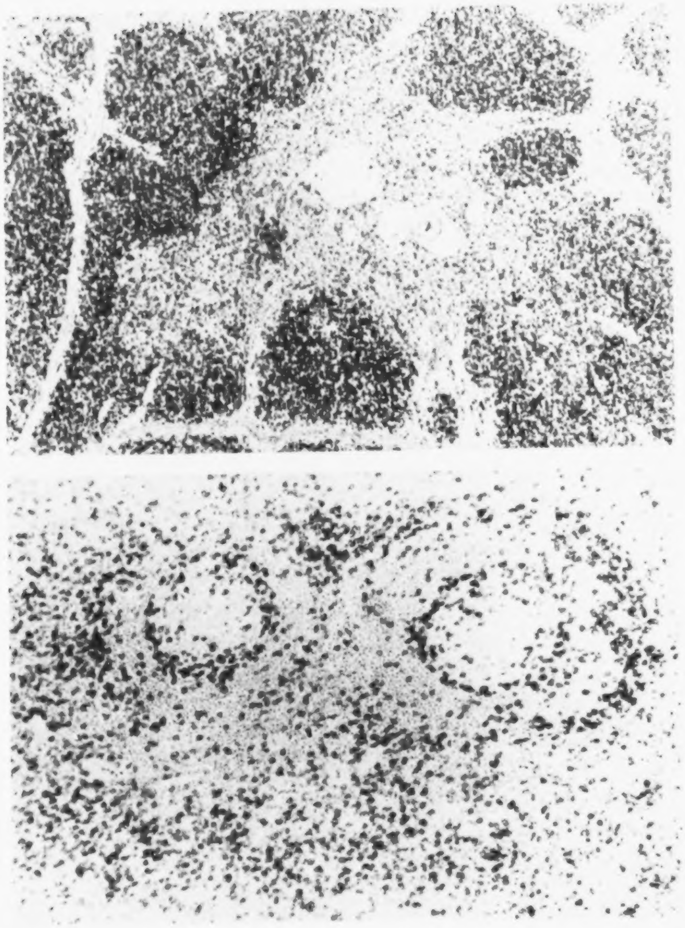


Fig.2
FACScan analysis of UCHL1 (c,d) and OPD4 (e,f) staining on peripheral blood lymphocytes (a,c,e) and monocytes (b,d,f). Controls are shown in a and b. Note the identical staining patterns for both antibodies on lymphocytes and the relative absence of monocyte staining for OPD4 (f) as compared to UCHL1 (d).



a. Thymus

b. Lymph Node

Fig.3
Immunoperoxidase staining with antibody OPD4 of thymus (a) and lymph node (b) demonstrating staining of the majority of cortical thymocytes and of proportions of medullary thymocytes in thymus (a) and of approximately half of the paracortical T lymphocytes in lymph node (b). Also note the relatively strong staining of germinal center T cells.

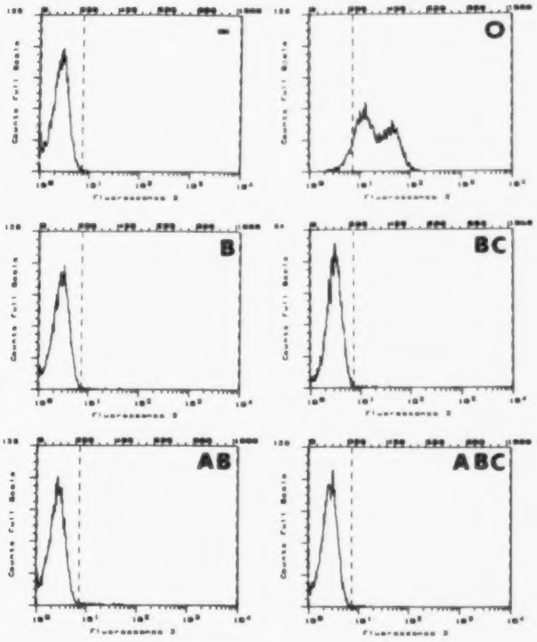


Fig.4
FACSCAN analysis of LCA transfectants stained with OPD4, A positive reaction is only found with clone A18 (top right) that is expressing none of the variable exons (CD45RO).

CHAPTER 6

BIOCHEMICAL ANALYSIS AND TISSUE DISTRIBUTION OF A SIALIC-ACID DEPENDENT CD45RC EPITOPE IN HUMANS

A version of this chapter has been submitted for publication.

Introduction

Leukocyte common antigen (CD45, LCA) represents a family of cell-surface glycoproteins with a molecular weight (MW) of around 200 kd expressed on nucleated hemopoietic cells [4, 31, 32]. The highly conserved intracytoplasmic domain of LCA is known to have tyrosine phosphatase activity [2,32] and is necessary for TCR signal transduction (8, 37). In contrast, the function of the extacellular domain that is highly variable in structure remains largely unclear. The variability is partly generated by alternate splicing of three variable exons (commonly labelled A, B, and C), such that LCA isoforms can express the protein sequences of either none, one, two or all of the three variable exons [13] . In addition, differential glycosylation is found in the common region of LCA and the variable region A (ie. CD45RA), B (ie. CD45RB) and the region that can only be detected when all the variable regions are not expressed (ie. CD45RO) [10, 23, 24]. These two levels of variation then create a great diversity of LCA variants being expressed on different cell types. In the past, due to the lack of anti-CD45RC reagents in humans, the expression profile of different LCA variants on lymphocyte subsets can only be deduced based on their reactivity with anti-CD45RA, -B and -O reagents. Although anti-CD45RC reagents for mouse and rats were available [7], only recently an anti-CD45RC reagent for humans was reported. Dianzani et al [5] suggested that antibody MCA.347 is an anti-CD45RC reagent, based on its unique reactivity and the fact that it is reactive with a 190 kd species not recognized by anti-CD45RA nor anti-CD45RB reagents. In this study, we examined this putative anti-CD45RC reagent in more detail. We first confirmed its reactivity with the variable region C using LCA transfectants and compared the tissue distribution of MCA.347 epitope with those of CD45RA, CD45RB and CD45RO.

Methods and Materials

1. Cells and LCA transfectant Cell Lines

Umbilical cord blood samples were obtained from informed healthy volunteers immediately after normal vaginal deliveries at a local hospital. Adult blood samples were obtained from healthy volunteers.

Mononuclear cells were isolated from the blood samples using Ficoll-Paque (Pharmacia, Uppsala, Sweden). The five transfectant cell lines and the control murine pre-B lymphocyte cell line were provided by Dr. M. Streuli. The establishment and characteristics of these lines have been described by Streuli et al [18].

2. Monoclonal Antibodies

Monoclonal antibody MCA.347 (rat IgG1) was purchased from Serotec. Anti-CD45RA Mab's MB1 and MT2 (IgG1), and anti-CD45RB Mab MT3 (IgG1), were previously produced in our laboratory and reported in detail elsewhere [8,11-13]. Mab KiB3 (anti-CD45RA's) was kindly provided by Dr. M. Parwaresch [4]. Anti-CD45RB Mab PD7/26 (IgG1) [24] and anti-CD45RO Mab UCHL-1 (IgG2a) [17,19] were kindly provided by Dr. D. Mason and Dr. P. Beverley, respectively. FITC conjugated avidin, FITC conjugated anti-CD45RA, PercP conjugated anti-CD3, -CD4 and -CD8, as well as PE conjugated anti-CD20, were all obtained commercially from Becton Dickinson. FITC conjugated rabbit anti-rat IgG, PE conjugated rabbit anti-mouse IgG1 and anti-mouse IgG2a were purchased from Southern Biotec.

3. Immunofluorescence stainings and flow cytometry

Isolated mononuclear cells were subjected to three-color immunofluorescence stainings and analysed by the FACScan (Becton Dickinson). After treatment with antibody MCA.347 for 30 minutes, PE conjugated rabbit anti-rat IgG antibody was applied for another 30 minutes at 4 C. Subsequently, cells were treated for 30 minutes with one of the followings: 1) FITC conjugated anti-CD45RA, 2) anti-CD45RB MT3, or 3) anti-CD45RO UCHL-1. FITC conjugated anti-mouse IgG1 and anti-mouse IgG2a were used to detect MT3 and UCHL-1, respectively. Following incubation with mouse serum to block the remaining anti-mouse antibodies for 15 minutes, the cells were finally exposed to one of the followings: PercP conjugated mouse anti-CD3, -CD4 ,-CD8 or -CD20 for 30 minutes. All steps were done at 4 C and three washes with phosphate-buffered saline (pH 7.5) were used between steps. With the FACScan, cell populations were then selected with gating based on size and forward scatter, along with positivity of CD3, CD4, CD8 or CD20.

4. Immunohistology

Lymph nodes, tonsils and spleens submitted for diagnostic reasons and fragments of normal thymus tissue, removed for technical reasons during cardiac surgery, were fresh frozen in OCT. Five micron thick frozen tissue sections were airdried and fixed in acetone for 15 minutes. The slides were incubated with MCA.347 in the dilution of 1:50 (20 ug/mL) for 30 minutes, followed by a second step incubation with peroxidase conjugated rabbit anti-rat Ig antibodies (Jackson ImmunoResearch), diluted 1:50, for 30 minutes. In between incubations, sections were washed in phoshate-buffered saline (pH 7.5) for 5 minutes. Peroxidase activity was visualized with 3-amino-9-ethylcarbazole (Sigma) and H2O2 for 15 minutes in the frozen sections. Nuclear counter-staining was obtained using Mayer's Hemalum.

5. Electrophoresis and Immunoblotting

Tonsillar mononuclear cells or peripheral mononuclear cells were lysed with extraction buffer containing 0.5% NP-40. After centrifugation, the supernatant was subjected to SDS-PAGE on a 5% gel according to the condition of Laemmli [7]. The electrophoretic transfer to nitrocellulose was performed according to the conditions of Towbin [22]. Immuno-stainings were performed with diluted supernatants as a first step, biotinylated goat anti-mouse (Dakopatts) or rabbit anti-rat Ig antibody (Vector) as the second step and peroxidase conjugated avidin (Dakopatts) as a third step incubation. Peroxidase enzyme staining was done with 3-amino-9-ethylcarbazole and H2O2 for about 10 minutes. TBS with Tween-20 was used to wash between steps. To test for sialic acid dependence of the MCA.347 epitope, immunoblots were incubated with neuraminidase (Sigma) at a concentration of 10 milliunits per mL of incubation buffer, at 37 C for 30 minutes. The subsequent stainings were carried out as above.

Results

1. Biochemical Analysis of the MCA.347 epitope

We first studied the identity of the MCA.347 epitope with LCA transfectants. As shown in fig. 1, MCA.347 were reactive with the clones D3.1 (ABC), B3.4.1 (BC) but not the others including the control mouse clone (E3), consistent with the anti-CD45RC identity. Using immunoblots prepared from lysates of unfractionated tonsillar cells, we tested which specific LCA isoforms could be recognized by MCA.347. Fig. 2 shows that MCA.347 recognized strongly the 220 kd species, and the 205 kd species with a lower intensity. At least two bands could be distinguished within the 205 kd range. The high 205 kd band was also recognized by PD7/26 (anti-CD45RB) and MT2 (anti-CD45RA), whereas the low 205 kd band was recognized by MT2 alone, suggesting that the high 205 kd band represents both AB and BC and the low 205 kd band represents AC. Staining with MCA.347 did not have detectable signal at the 190 kd level with lysates prepared from tonsillar cells, which were predominantly B cells. With lysates prepared from peripheral blood mononuclear cells which were rich in T cells, MCA.347 recognized three bands at 220, 205 and 190 kd. The low 205 kd species (ie. AC) was not detectable. After treating the immunoblots with neuraminidase for 30 minutes at 37 C, reactivity of MCA.347 was largely eliminated, indicating that MCA.347 epitope is sialic acid-dependent (fig.2).

2. Tissue Distribution of MCA.347 epitope

We examined the tissue distribution of the MCA.347 epitope with the routine immunoperoxidase staining method. As shown in fig. 3a, cortical thymocytes were largely negative with MCA.347 staining. In contrast, medullary thymocytes were heterogenously positive. A few B cell follicles inside the medulla strongly reactive with MCA.347 were also noted. In reactive lymph nodes / tonsils (fig.4a), MCA.347 was reactive with the mantle zones and inter-follicular B cell areas. Germinal center cells were negative. A significant proportion of T cells in the interfollicular areas was also negative. The positivity within the mantle zones found in both lymph nodes and tonsils was heterogeneous, with the presence of MCA.347 -dim and -bright cells (fig. 4b). The lymphoid follicles in spleens followed the same reactivity patterns. The marginal zones found uniquely in spleens were uniformly positive with MCA.347 staining (not shown).

In cord peripheral blood, MCA.347 was reactive with B cells homogenously, shown in fig.5 as a single narrow peak. In contrast, an additional MCA.347 dim population was identified in the $CD3^+$ population. When we studied

CD4 and CD8 cells separetely, most of the MCA.347^{dim} cell population were in the CD8^{+} cell subset. When compared to the adult peripheral lymphocytes, a few postnatal changes were observed. First, B cells could be readily divided into MCA.347 -bright and -dim subsets and the expression of MCA.347 epitope was directly proportional to CD45RA expression (fig. 6a). Second, the expression of MCA.347 epitope was also directly proportional to that of CD45RA within the CD3^{+} subset, with about 30-40% of peripheral CD3^{+} T cells being MCA.347 dim or negative (fig.6b). As expected, UCHL-1 (anti-CD45RO) stained inversely proportional to MCA.347. Double stainings with MCA.347 and anti-CD45RB revealed three cell groups in both CD4 and CD8 subsets: 1) RB^{2+}MCA.347^{2+}, 2) RB^{2+}MCA.347^{+}, and 3) RB^{+}MCA.347^{-} (fig.7a). Peripheral CD8^{dim} cells were CD45RA^{2+} and MCA.347^{dim} (fig.7).

Discussion

One of the feature of LCA is the great structural variation in the extracellular domain, generated by alternate splicing of mRNA and differential glycosylation. It has been postulated that the extracellular domain of LCA is responsible for the distribution of the tyrosine phosphatase activity embeddied in the constant cytoplamsic domain. Evidence is accumulating in support of this model. Using immunofluorescence co-capping technique, Dianzani et al 1992 [3] showed the specific associations between LFA-1 and CD45RA, CD2 and CD45RO, as well as CD4/8 and CD45RC on peripheral T lymphocytes from human adults. Recently, it was also shown that the extracellular domain is not necessary for coupling TCR and transmembraneous signaling [6,23], and logically it carries some other important functions such as ligand selection. Therefore, it is crucial to have a complete profile of LCA isoforms expressed on cells of different functional or developmental stages. Previous works have shown that LCA isoforms with higher MW (ie. 220 and 205 kd) are expressed on medullary thymocytes, B and virginal T cells whereas those with lower MW (ie. 190 and 180 kd) are expressed on cortical thymocytes and memory T cells [2,20.21]. However, further detail is difficult to obtain due to the lack of anti-CD45RC reagents. This report on the anti-CD45RC reagent MCA.347 thus provides a more comprehensive picture of CD45 isoform expression.

The reactivity pattern of MCA.347 with CD45 transfectants is consistent with an anti-LCA antibody, as it is reactive with all clones carrying the variable region C but not those without. The MCA.347 epitope is largely composed of carbohydrates as shown by its sensitivity to neuraminidase treatment. Using lysates prepared from predominantly tonsillar B cells, MCA.347 is reactive with the 220 kd species (ABC), and more weakly with the high 205 kd species (AB and BC) and low 205 kd species (AC). To our knowledge, this is the first report on the existence of AC. Since this low 205 kd species (AC) is absent with the lysates prepared from predominantly peripheral T cells, its expression may be confined to the B cell lineage. Consistent with the previous data [3], MCA.347 recognizes a 190 kd species (C) from lysates of peripheral T cells but not B cells. Since the expression of the MCA.347 is largely confined to the CD45RA$^+$ T cells, this LCA isoform (C) is expected to be found on the same cell population (ie. virginal T cells).

Generally, the MCA.347 epitope is expressed almost directly proportional to that of CD45RA, as examplified by cortical and medullary thymocytes, peripheral T and B cells, monocytes, mantle and marginal zone B cells. Together, these results suggest that the variable exon C is largely expressed in association with exon A, such as in the form of ABC and AC. Isoforms AB, BC and C are therefore not predominant species being expressed on these cell types. However, there are two exceptions to this general rule: 1) Although the germinal center B cells express predominantly the 220 kd band (ABC), they do not express the MCA.347 epitope. The explanation is most likely related to the hyposialylated state of germinal center cells which is also known to alter the expression of the LCA common determinant (CD45), CD45RA and CD45RB [8]. This hyposialylated state may be crucial since the lack of sialic acid on cell surface facilitates recognition by phagocytes and hence destruction of apoptotic cells [16]. 2) Peripheral CD8^{dim} cells (including the natural killer cells, which also express predominantly the 220 kd isoform (ABC), are CD45RA^{2+} RB^{2+} but only MCA.347^{dim}. This again may reflect a selective hyposialylated state of the variable C, or alternatively, a selective strong expression of a LCA variant which express A and B without C (ie. AB).

There are significant postnatal changes of CD45RC expression. One example is that cord blood CD4$^+$ cells are largely MCA347^{2+} cells whereas their adult counterparts can be distinguished into at least three subpopulations:

(1) CD45RA^{2+} RB^{2+} RC^{2+} RO^{-}, (2) CD45RA^{-} RB^{2+} RC$^{-/+}$ RO^{+}, (3) CD45RA^{-} RB^{+} RC^{-} RO^{+}. The stronger expression of CD45RB over either CD45RA and CD45RC in subset (2) and (3) suggests that it is due to the presence of isoform B (ie. 190 kd) instead of BC. This correlates well with the fact that CD4^{+} cells are strongly reactive with anti-CD45RB mab MT3 which largely recognizes the variable region B in the absence of A or C [12]. Similar postnatal changes of CD45RC expression in B cell lineage are also evident, when comparing MCA.347^{2+} cord blood B cells with germinal center B cells (MCA.347^{-}) and a proportion of peripheral blood B cells which express higher level of CD45RA, RB and RC. Significant postnatal changes are observed in other variable regions of LCA (9), and these modifications as a whole most likely are results of antigenic challenge and/or immunologic maturation. Most of the cord blood as well as adult peripheral blood CD8^{+} cells (80-90%) are CD45RA^{2+} RB^{2+} RC^{2+}, suggesting the LCA variant ABC as being the most predominant species expressed without significant postnatal changes in this cell type.

In conclusion, we confirmed that MCA.347 is a anti-CD45RC Mab which recognizes a sialic-acid dependent epitope. Differential glycosylation is found in the variable region C, confirming that the regulation of the carbohydrate is the rule rather than exception, and that it provides an important regulatory mechanism for ligand selection. The results from the tissue distribution of MCA.347 epitope allow us to have a better understanding of the LCA expression. Based on the results and discussion mentioned, a comprehensive LCA expression profile on various lymphoid cells is proposed (Table 1). Since individual LCA isoforms could have different ligand preference, further studies on LCA should be focused on specific isoforms instead of variable regions which can be shared by more than one isoform.

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Table 1. Proposed CD45 Expression Profile on Lymphoid Cells

1. Thymocytes							
a. Cortical	-	-	-	-	B	-	O
b. Medullary	**ABC**	AB	BC	-	B	C	-
2. Peripheral T cells							
a. virginal T	**ABC**	AB	BC	-	B	C	-
b. memory T	-	-	-	-	**B**$^{+/-}$	-	**O**
3. B cells (*)	**ABC**	AB	BC	AC	-	-	-
4. peripheral CD8^{low}(*)	**ABC**	AB	BC	-	-	-	-

Footnotes

1. (*) indicates the downregulation of MCA.347 epitope due to hyposialylation in germinal center B cells and peripheral CD8^{low} cells.
2. B$^{+/-}$ indicates the presence of RB^{high} and RB^{low} memory T cells.
3. The bold LCA isoforms represent the predominant species.

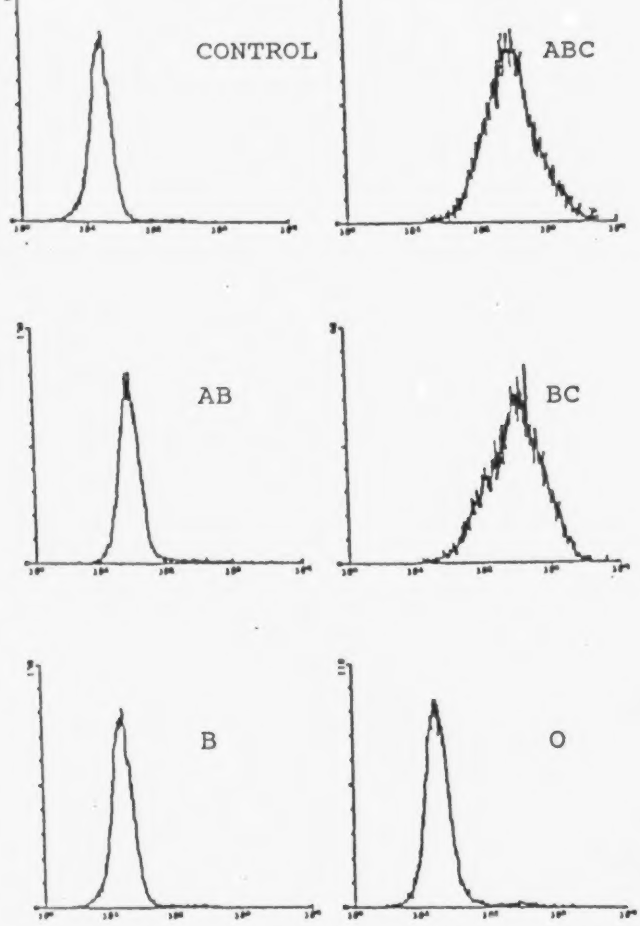


Fig.1
FACSCAN analysis of LCA transfectants with MCA.347. The antibody shows positive staining with tranfectants expressing ABC and BC, but negative staining with the control cells and transfectants expressing AB, B and O. This indicates that MCA.347 is reactive with an epitope associated with the C exon of LCA.

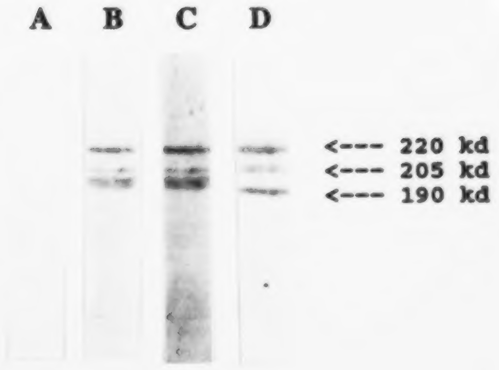


Fig.2
Immunoblots of unfractionated tonsillar lymphoid cells. Lane A shows the staining of MCA.347 is abrogated after neuramindase pretreatment of the immunoblot. Note the identical staining pattern of MT2 (anti-CD45RA) (lane B) and MCA.347 (lane C), with two distinct bands within the 205 kd region. Lane D is the immunoblot stained for 6G3 (anti-CD45RB) which has a strong 190 kd band and only one 205 kd band.

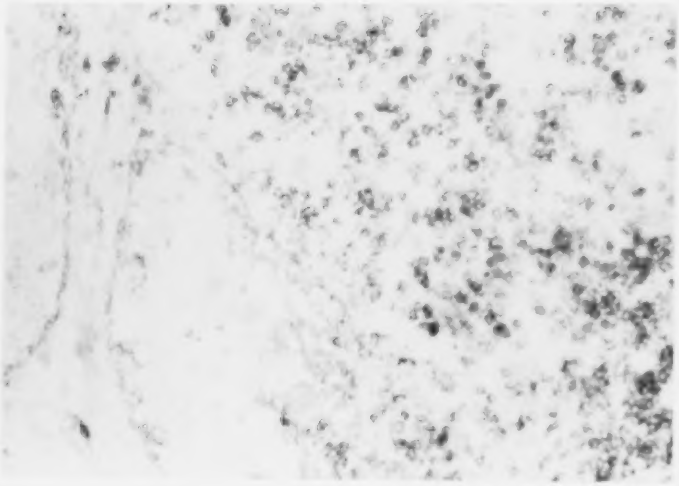


Fig.3a
Immunoperoxidase staining of human thymus with MCA.347. MCA.347 does not react with cortical thymocytes (left) but heterogenously reacts with medullary thymocytes (right), showing dim- and bright- stained cells.

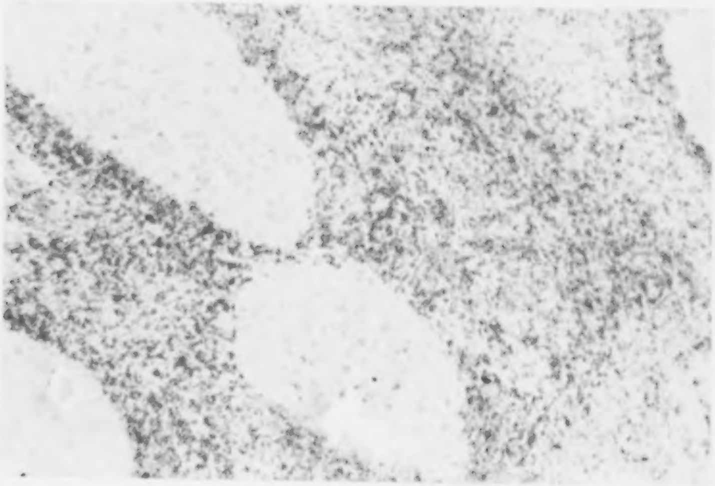


Fig.3b
Immunoperoxidase staining of human tonsils with MCA.347. Note negative staining of MCA.347 in germinal centers. A proportion of the interfollicular T cells are also negative (left).

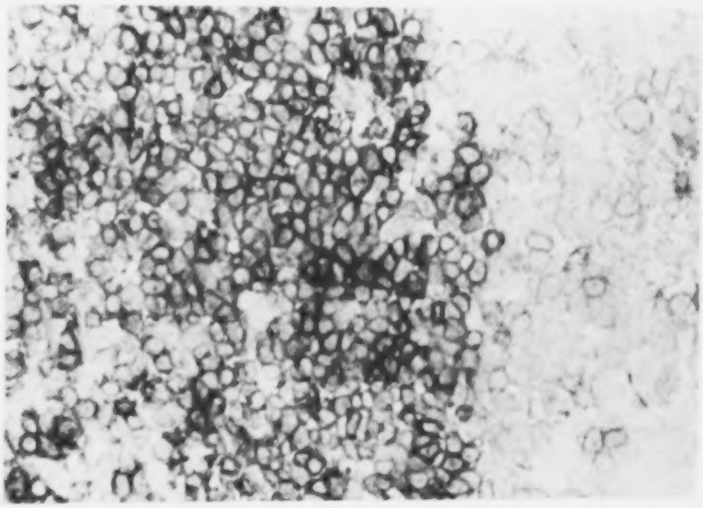


Fig.3c
High magnification of the same follicle shown in fig.3b, showing the negative staining of germinal center cells (right), and the variable staining with mantle zone cells with dim and bright subsets.

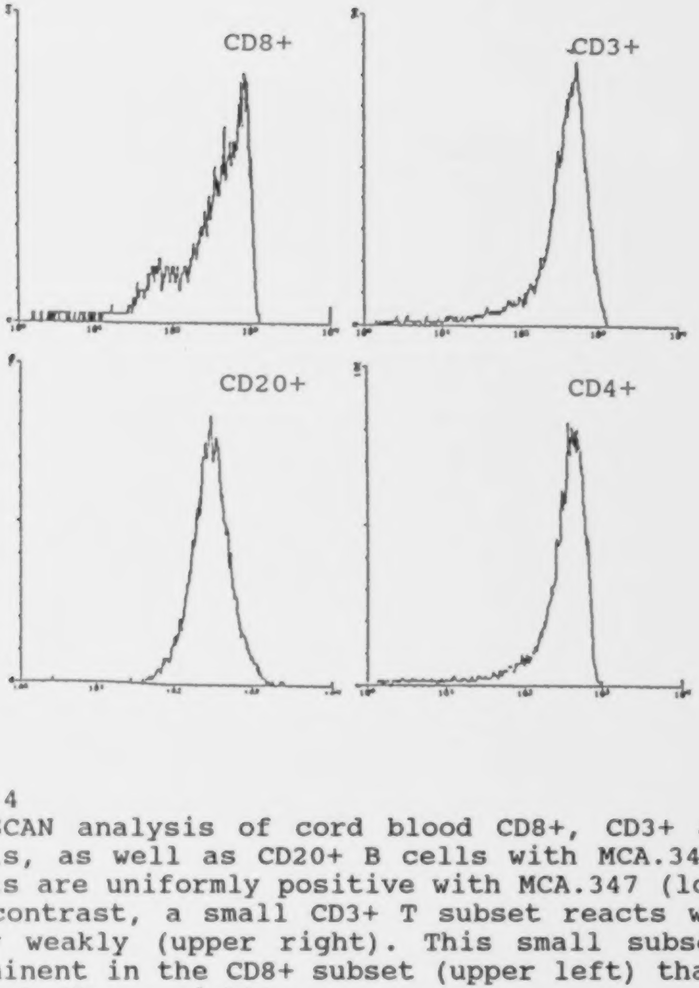


Fig.4
FACSCAN analysis of cord blood CD8+, CD3+ and CD4+ T cells, as well as CD20+ B cells with MCA.347. CD20+ B cells are uniformly positive with MCA.347 (lower left). In contrast, a small CD3+ T subset reacts with MCA347 only weakly (upper right). This small subset is more prominent in the CD8+ subset (upper left) than the CD4+ subset (lower right).

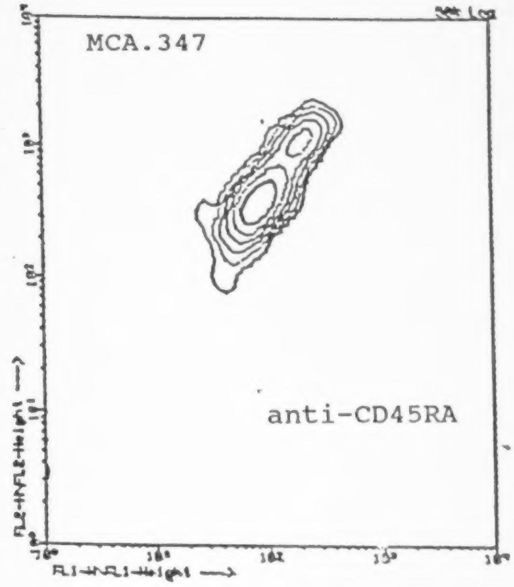
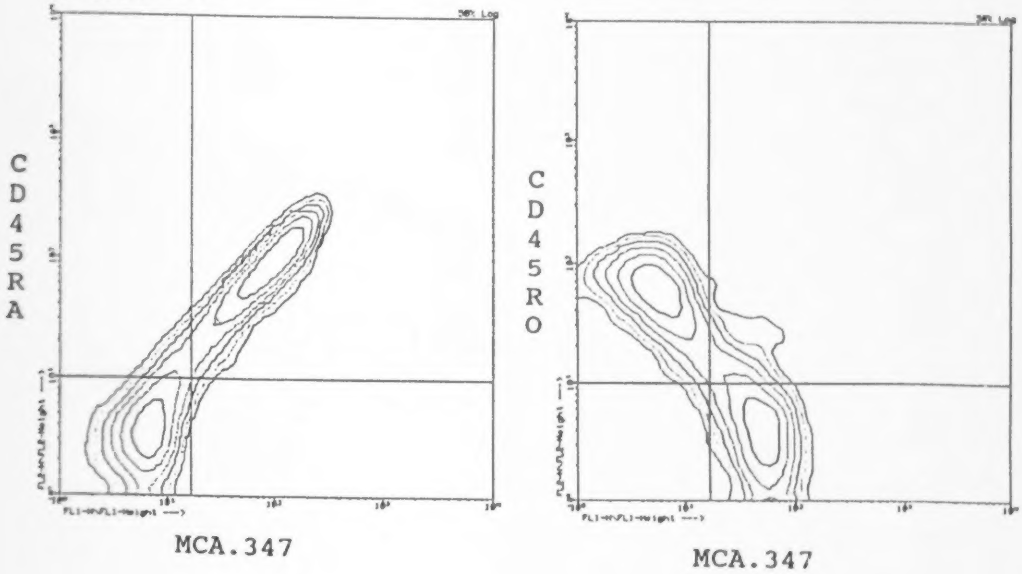


Fig.5
Double staining of peripheral CD20+ B cells with MCA.347 and MB1 (anti-CD45RA) reveals dim and bright subsets. The expression level of MB1 is directly proportional to that of MCA.347.



A

B

Fig.6
Double stainings of peripheral CD3+ T cells with MB1 (anti-CD45RA) (A) or UCHL-1 (anti-CD45RO) (B) with MCA.347. The expression level of MCA.347 is directly proportional to that of MB1 but inversely proportional to that of UCHL-1.

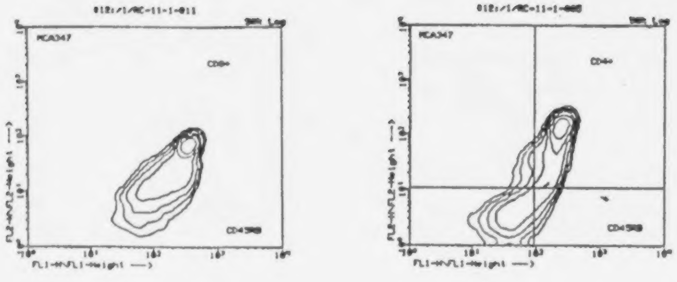


Fig.7
Double stainings of MCA.347 with CD45RB in CD4+ and CD8+ T-cell subsets. In addition to the double positive and negative populations, a MCA.347^{dim}CD45RB^{bright} subset can be observed.

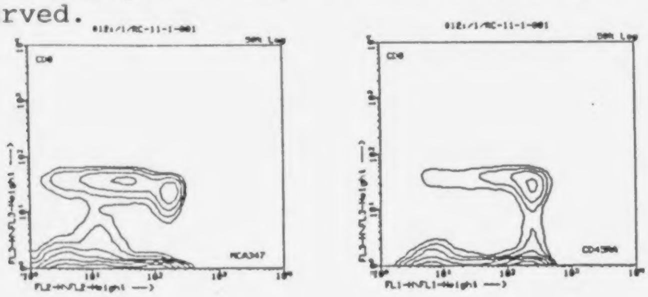


Fig.8
CD8^{dim} cells express low level of MCA.347 but high level of CD45RA.

CHAPTER 7

POSTNATAL CHANGES OF CD45 EXPRESSION IN PERIPHERAL BLOOD T AND B CELLS

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Introduction

Leukocyte common antigen (LCA, CD45) is a family of leukocyte cell surface molecules expressed on all nucleated hemopoietic cells [4,27]. LCA has a constant cytoplasmic domain which embodies tyrosine phosphatase activity [2,6]. The structure of the extracellular domain is highly variable and the heterogeneity is generated by alternate splicing of mRNA [10,26] and by differential glycosylation (7,20]. Expression of different LCA isoforms has served as a marker for different functional T cell subpopulations [reviewed in 4]. The best-documented is the expression of $CD45RA^+$ (220/205 kd) on virginal $CD4^+$ T cells and CD45RO (180 kd) on $CD4^+$ memory cells. Some of the evidence supporting this concept derives from the study of cord blood lymphocytes which are immunologically naive and are largely $CD45RA^+$ and $CD45RO^-$ [3,13]. With a panel of anti-LCA monoclonal antibodies (mab's) reacting with carbohydrate- or protein- specific epitopes, we have previously shown that differential glycosylation of the three LCA variable regions (commonly labelled A, B and C) is fine-controlled and creates extensive additional heterogeneity [7]. These specific LCA glycosylations may have an important role in determining the interaction with ligands and thereby directing the tyrosine phosphatase activity embedded in the constant domain to the appropriate targets. In this study, we compare LCA expression on cord blood lymphocytes and their adult counterparts. The results indicate several postnatal changes of LCA glycosylation.

Methods and Materials

1. Cells

Umbilical cord blood samples were obtained from informed healthy volunteers immediately after normal vaginal deliveries at a local hospital. Pediatric peripheral blood samples were collected from afebrile patients who were admitted in hospital for elective surgery. They ranged from 9 months to 16 years of age and all of them had normal complete blood counts and differentials. Adult peripheral blood samples were received from healthy volunteers (aged from 22 to 60). Mononuclear cells were isolated from the blood samples using Ficoll-Paque (Pharmacia, Uppsala, Sweden).

2. Monoclonal Antibodies

Anti-CD45RA Mab's MB1 and MT2 and anti-CD45RB Mab MT3 were previously produced in our laboratory and reported elsewhere [7,15-17]. Mab KiB3 (anti-CD45RA's) [5] was kindly provided by Dr. Parwaresch. Anti-CD45RB PD7/26 [7,19,20,24,30] and anti-CD45RO (UCHL-1) [23,25] were kindly provided by Dr. David Mason and Dr. Peter Beverly, respectively. FITC conjugated goat anti-mouse Ig, PercP conjugated anti-CD3, PE conjugated anti-CD5 as well as PE conjugated anti-CD20 were obtained commercially from Becton Dickinson.

3. Staining and Flow Cytometry

The cells were analysed by two-color immunofluorescence on a FACScan (Becton Dickinson). After the treatment with the primary mabs at 4°C for 30 minutes, FITC conjugated goat anti-mouse Mab was applied for another 30 minutes at 4°C. Following incubation with mouse serum to block the remaining goat anti-mouse antibodies for 15 minutes, the cells were exposed to anti-CD20 coupled with PE or anti-CD3 coupled with PercP. Three washes with phosphate-buffered saline (pH 7.5) were performed between steps. For analysis, cell populations were gated based on size and foward scatter, along with positivity for CD3 or CD20.

Results

A total of six umbilical cord, four pediatric, and five adult peipheral blood samples were analysed. Only representative results were shown. Results from the analysis of CD45RA expression in $CD3^+$ cord blood T cells are shown in figure 1. Anti-CD45RA reagents MB1 and MT2 were positive with over 90% of the cord blood T lymphocytes, with stronger MT2 than MB1 staining (fig. 1a,b). In contrast, less than 5% of cord blood T cells were stained weakly with anti-CD45RO UCHL-1. Double staining with UCHL-1 and MB1 confirmed the existence of a single $MB1^+/UCHL\text{-}1^-$ T cell population (fig. 2a). Staining with anti-CD45RA reagent KiB3 was largely negative (fig. 1c). As expected, MB1 and MT2 were strongly reactive with a variable proportion of adult T cells depending on the age of the individual, for instance around 80% in a 3 year old and 60% in a 27 year old individual. In addition to the CD45RA^{bright} and the CD45RA^{-} T cell subsets, there was a CD45RA^{dim} T cell population recognized by both MB1 and MT2 (fig. 1d,e). Double staining with UCHL-1 and MB1 showed that this CD45RA^{dim} T subset was also UCHL-1^{dim} (fig. 2b).

There were two other significant differences in CD45RA expression between the cord blood and adult T cells. The first difference is that adult CD45RA^{bright} T cells were characterized by higher MB1 and lower MT2 expression than their cord blood counterparts. The highest anti-CD45RA staining intensity with cord blood lymphocytes (ie. MT2) was largely similar to that with adult blood lymphocytes (ie. MB1), reflecting that LCA isoforms containing the variable region A such as ABC (220 kd) and AB (205 kd) remained largely constant. This difference in CD45RA expression diminished in the early postnatal life (3 year). In fact, the expression level of MT2 was virtually equal to that of MB1 in late adolescent years (ie. 16 year). Therefore, the transition from MT2 high to MB1 high is a gradual process. The second difference is that, in contrast to cord blood T cells, 15-20% of the T cells in adults were reactive with KiB3 cells and the staining intensity of this KiB3^{+} population was quite heterogeneous (fig. 1f). This KiB3^{+} T cell subset was not prominent in the pedriatric group (results not shown).

With the same gating protocol for CD3^{+} cells, analysis of the CD45RB expression was carried out with MT3 and PD7/26. All cord blood T cells were homogeneously reactive with these two anti-CD45RB reagents, as shown by the narrow peaks (fig. 3a,b). The staining intensity and pattern of PD7/26 was largely similar in adult, pediatric and cord blood T cells (fig. 3 c,e). In contrast, only the adult T cells showed two distinct subsets with MT3 staining, namely MT3^{dim} and MT3^{bright} (fig 3d). The cord and pediatric blood T cells had a MT3 staining intensity equivalent to that of MT3^{dim} adult T subset. Staining with PD7/26 did not result in a clear distinction of CD45RB^{bright} and CD45RB^{dim} in adults.

We next studied the LCA expression on the CD20^{+} B lymphocytes. All three anti-CD45RA reagents, MB1, MT2 and KiB3 were uniformly reactive with B cells in cord blood, although the level of KiB3 was lower than those of the other reagents (figure 4 a,b,c). In general, the CD45RA expression on adult B cells was slightly higher than on their cord blood counterparts (fig. 4 d,e,f). Two prominent postnatal changes are noted: (1) the KiB3 epitope was more strongly expressed (figure 3 f); (2) MB1 was differentially expressed on adult B cells, as shown by the presence of two distinct B subsets: $MB1^{dim}$ and $MB1^{bright}$. The adult $MB1^{dim}$ was equivalent to the cord blood B cells in staining intensity. These two subsets of B cells can only be distinguished in late adolescent years (ie. 16 year) but not early postnatal life (ie. 3 year).

The CD45RB reagent PD7/26 was positive with all B cells in cord, pediatric and adult blood B cells with approximately the same high staining intensity, with no distinction of $CD45RB^{bright}$ or $CD45RB^{dim}$ subsets (fig. 5). In contrast to PD7/26, adult B cells could be readily divided into $MT3^{dim}$ and $MT3^{bright}$ subsets. The cord blood B cells had a staining intensity similar to the $MT3^{dim}$ adult B cells. This distinction can be seen in early postnatal life (ie. 9 month) and the $MT3^{bright}$ cell increased in percentage with age, such as 10% in a 9 month old and 25% in a 16 year old. We also studied how $MT3^{bright}$ and $MT3^{dim}$ adult B cell subsets relate to the $CD5^+$ B cell subset in adults. Figure 6 shows that the $CD5^+$ adult B cells were largely confined to the $MT3^{dim}$ B subset, whereas virtually all cord blood B cells are $CD5^+$ and $MT3^{dim}$.

Discussion

Diversity of LCA expression is generated by differential glycosylation, in addition to the variation due to differential splicing in the protein backbone. Therefore, different anti-CD45RA Mabs have different reactivity patterns. The same principle also applies to anti-CD45RB Mab's MT3 and PD7/26 [7].

In this study we analyzed the LCA expression on lymphocytes from cord blood with the same panel of anti-CD45R mabs, and compared the results with those in pediatric and adult peripheral blood cells. It has previously been shown that cord blood T cells have low expression of CD45RO, LFA-1 and CD29 (integrin ß1) which are considered markers for memory T cells [12,22]. Functionally, cord blood T cells (mostly CD45RA+) also respond different from adult $CD45RA^+$ T cells: the $CD45RA^+$ T cells from cord blood are predominantly suppressive for

Ig production [3] and show impaired responsiveness to stimulation with anti-CD2 and anti-CD3 [14].

As cord blood lymphocytes are immunologically naive and immature, a comparison with the peripheral blood cells in postnatal life can provide information on the maturation of lymphocytes. One of the established postnatal changes of LCA expression is the expansion of $CD45RO^+$ memory T cells and the decline of $CD45RA^+$ virginal T cells [13]. Furthermore, the $CD45RA^{dim}/CD45RO^{dim}$ T cells (labelled transitional T cells) which reflect recently stimulated T cells in vivo exist only postnatally.

With the unique specificities of the anti-LCA Mab's employed in this study, we demonstrated additional modifications of LCA expression generated by differential glycosylation during the postnatal period. Variable regions A and B remain largely constant quantitatively, and the patterns of these modifications is too complex to be explained by LCA mRNA alternate splicing. One example is that $CD45RA^{bright}$ adult T cells appear to have a higher MB1 but lower MT2 expression than $CD45RA^{bright}$ cord blood T cells. Results from the pediatric group show that this change of CD45RA expression is a gradual process. As the MT2 epitope is sialic-acid dependent and partial deglycosylation of LCA can uncover the protein epitopes as recognized by MB1 and KiB3 [7], this finding suggests that the LCA isoforms found on adult blood T cells are less heavily sialylated on the CD45RA variable region and hence less negatively charged. In contrast to the adult peripheral blood T cells, there is a subset of medullary thymic T cells in adults which have a higher expression of MT2 than MB1 [7]. Together, such variations in glycosylation are selectively controlled and may influence the nature of ligand interactions.

Similar principles apply to the CD45RB variable region recognized by MT3 and PD7/26, both of which recognize sialic acid-dependent epitopes [16,20]. Since MT3 expression changes postnatally in B and T cells whereas PD7/26 expression is constant, it is possible that the MT3 epitope plays a role in determining specific LCA-ligand interactions. This is supported by a study showing that MT3 mab has the ability to inhibit mixed leukocyte reactions [8].

Several lymphocyte subsets appear to develop only postnatally: $KiB3^+$ T cells are present only in adult life. It is possible that the KiB3 epitope is only accessible on T cells which are antigen-experienced.

Since some CD45RA T cells retain CD45RA upon stimulation in vitro [21], and since there are transitional T cells which coexpress CD45RA and CD45RO in vivo [18,31], KiB3^{+} T cells may represent a subpopulation of memory CD45RA^{+} T cells and/or transitional T cells. In fact, the proportion of the KiB3^{+} T cells increases with age, suggesting loss of sialic acid-dependent components (results not shown). An alternative possibility is that KiB3^{+} T cells represent a distinct lineage unrelated to the switch from CD45RA^{+}CD45RO^{-} to CD45RA^{-}CD45RO^{+} after activation. The fact that KiB3^{+}T cells is not evident until in adulthood favor the latter possibility.

CD45RB^{bright} T cells as shown with MT3 staining also develop in adult life. The existence of this subpopulation has been reported in mouse [1,9], rats and humans [11], but the function of CD45RB^{bright} and CD45RB^{dim} T subsets has not been settled. In humans, it was postulated that CD45RB^{bright} T cells represent TH1 cells whereas the CD45RB^{dim} T cells comprise TH2 cells [11]. In consistent with this, T cells collected from synovium affected by Rheumatoid Arthrits are enriched in CD45RB^{dim} CD45RO^{+} cells which have potent B-helper activity [28]. Future studies on the cytokine production from cord blood T cells will provide more insight in this regard. Nevertheless, CD45RB^{bright} T cells represent an additional immune component which may help constitute the more balanced and better-equipped immune system in adult than the neonates.

The expression of LCA on B cells has not been studied as extensively as that on T cells. In addition to MB1, staining with MT3 but not PD7/26 shows the distinction of two B subsets. MT3^{bright} B cells may represent antigen-experienced memory B cells or a distinct functional B subset. Different from the MB1^{bright} cells, MT3^{bright} B subset is present in early postnatal life suggests that these cells carry crucial function in a relative immature immune system. In accordance with this, splenic marginal zone cells that are the major population of MT3^{bright} B cells in lymphoid tissues appears at around the age of two [29]. The MT3^{bright} B cells are characteristically CD5^{-}. CD5^{+} B cells in adults are included in the MT3^{dim} B subset, similar to the majority of cord blood B cells.

In conclusion, LCA isoforms show significant differences in glycosylation between pre- and post-natal life. Several lymphocyte subpopulations with unique LCA glycosylations, are found only postnatally and may be the products of antigenic encounters. An alternate possibility is that they represent distinct lines of differentiation which expand postnatally. In either case, these results provide evidence that the glycosylation of CD45 is finely controlled and crucial to the maturation of the immune system.

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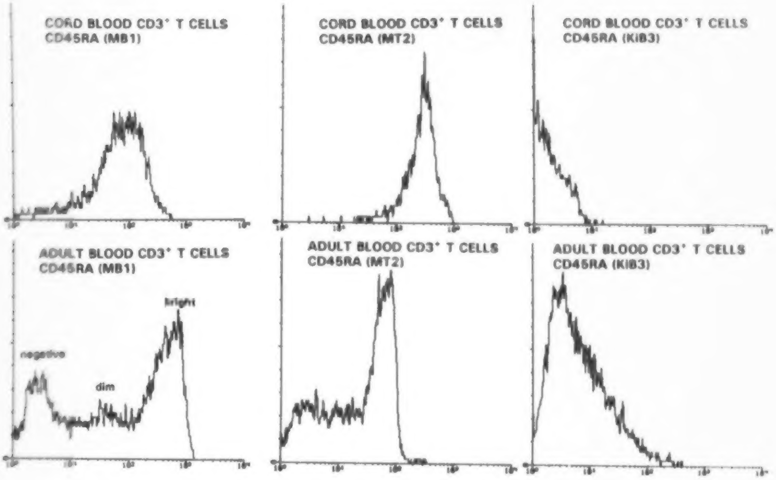

Fig.1
FACScan analysis of cord blood $CD3^+$ T cells (upper row) and peripheral blood $CD3^+$ T cells (lower row) with CD45RA reagents (left: MB1; middle: MT2; right: KiB3). The vast majority cord blood T cells are $MB1^+$, and are strongly $MT2^+$, whereas they are $KiB3^-$. The peripheral blood T cells show $MB1^-$, $MB1^{dim}$ and $MB1^{bright}$ T cells. MT2 shows similar, but overall weaker staining than MB1. MT2 is much weaker, whereas KiB3 is stronger than in the cord blood cells.

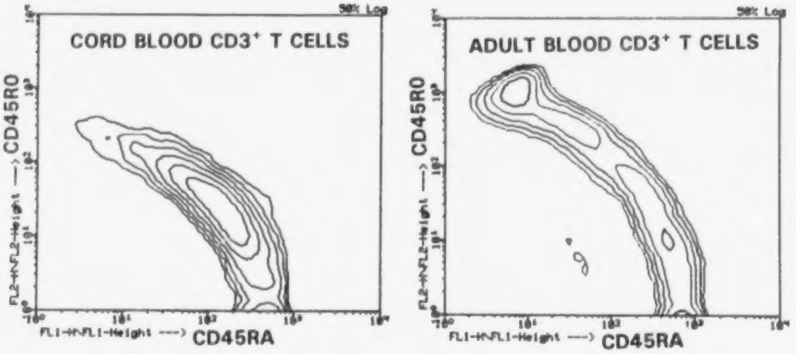

Fig.2
FACScan analysis of cord blood CD3$^+$ T cells (left) and adult peripheral blood CD3$^+$ T cells (right) with double stainings of UCHL-1 (CD45RO) and MB1 (CD45RA). The cord blood cells are largely CD45RO$^-$ and CD45RA$^+$. In contrast, adult blood cells can be divided into (1)CD45RA^{dim} CD45RO^{bright}, (2) CD45RA^{dim} CD45RO^{dim}, and (3) CD45RA^{bright} CD45RO^{bright}.

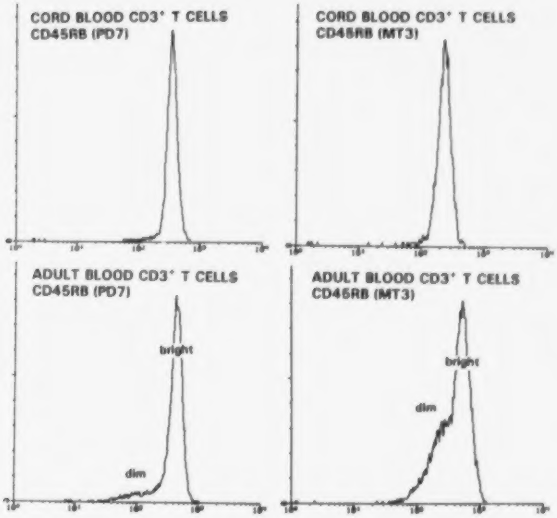

Fig.3
FACScan analysis of cord blood CD3$^+$ T cells (upper row) and peripheral blood CD3$^+$ T cells (lower row) with CD45RB reagents (left: PD7; right MT3). The cord blood cells show uniform strong staining for PD7 and MT3. The peripheral blood cells show a relatively small PD7/26^{dim} population, but the majority of the cells stains identical to the cord blood cells. However, with MT3 there is an additional MT3^{bright} population that stains stronger than the MT3^{dim} population that has a staining intensity that is similar to the cord blood cells.

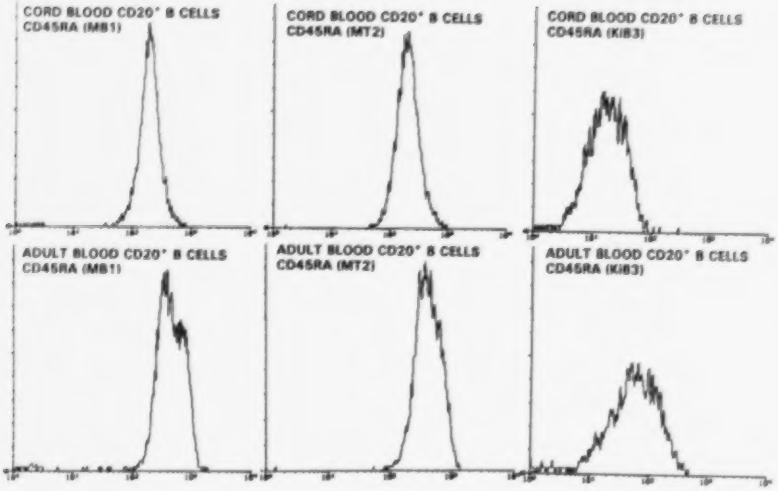

Fig.4
FACScan analysis of cord blood CD20$^+$ B cells (upper row) and peripheral blood CD20$^+$ B cells (lower row) with CD45RA reagents (left: MB1; middle: MT2; right: KiB3). Cord blood B cells show uniform positive staining for MB1 and MT2, whereas they show weaker staining for KiB3. Staining for MB1 is differential on adult blood B cells with an indication of two subsets. MT2 staining is uniformly and similar between adult and cord blood cells. On the other hand, KiB3 staining is stronger in the peripheral blood cells.

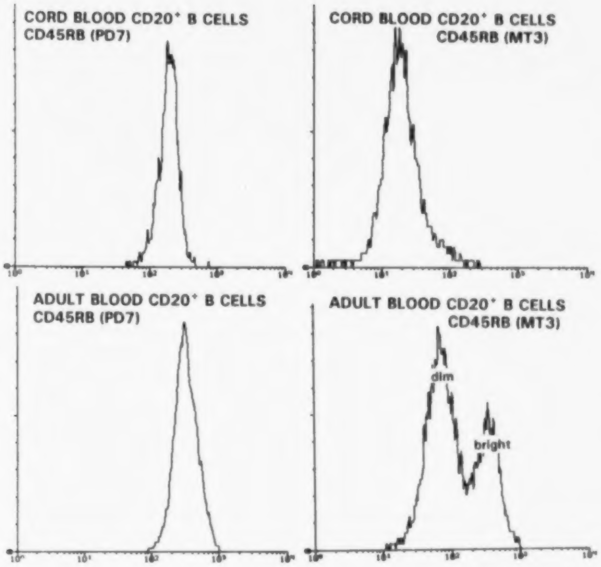

Fig.5
FACScan analysis of cord blood CD20$^+$ B cells (upper row) and peripheral blood CD20$^+$ B cells (lower row) with CD45RB reagents (left: PD7; right: MT3). Cord blood B cells show strong uniform staining for PD7 and weaker staining for MT3. Adult blood B cells have slightly stronger PD7 staining and show a bimodal staining pattern in which both the MT3^{dim} and MT3^{bright} populations stain stronger than the cord blood B cells.

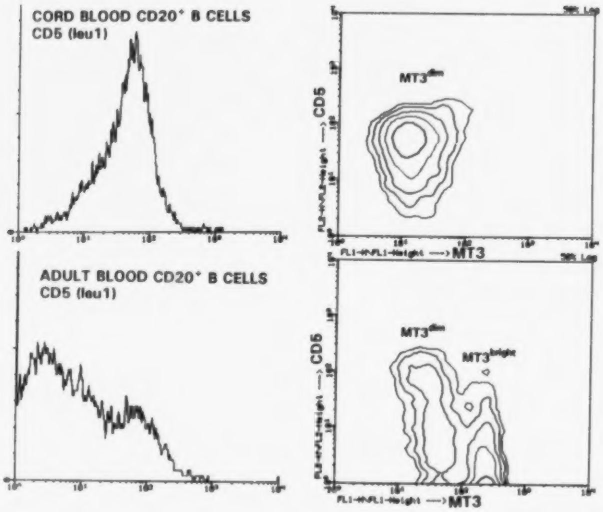

Fig.6
FACScan analysis of cord blood CD20$^+$ B cells (upper row) and peripheral blood CD20$^+$ B cells (lower row) for CD5 (left) and for CD5 (FL2 channel) versus MT3 (FL1 channel) (right). Virtually all CD20$^+$ cord blood B cells express CD5 and are MT3^{dim}, CD5$^+$. The peripheral blood CD20$^+$ cells show a subpopulation of CD5$^+$ cells that are MT3^{dim}, although they stain somewhat brighter than cord blood CD5$^+$ cells.

CHAPTER 8

CHARACTERIZATION OF A DISTINCT HUMAN B CELL SUBSET EXPRESSING HIGH LEVEL OF CD45RB CARBOHYDRATE EPITOPES

A version of this chapter is in preparation for publication.

Introduction

Leukocyte common antigen (CD45/LCA/T200) is a family of cell surface glycoproteins expressed on nucleated hemopoietic cells (Thomas 1989). Its constant cytoplasmic portion possesses tyrosine phosphatase activity which has been shown to play important roles in lymphocyte activation (Mustelin 1989, Shiroo 1922, Sieh 1993, Volerevic 1992). Heterogeneity of the LCA extracellular domain is generated by both differential glycosylation and alternate splicing of mRNA (Thomas 1988, Lefrancois 1986). Various LCA isoforms are expressed on lymphocytes in a finely-controlled manner closely linked to differentiation. One example is the expression of CD45RO (190 kd) on memory T cells and CD45RA (220 and 205 kd) on virginal T cells (Serra, Akbar). LCA expression on B cells is much less studied than T cells, although it is known that they express predominantly the two LCA species of higher molecular weight at 220 and 205 kd (Coffmann). We previously studied the LCA expression on human B cells with a panel of anti-LCA monoclonal antibodies which reacted with either carbohydrate- or protein- associated epitopes (Lai). Our results show that differential glycosylation of LCA creates great diversity of LCA expression on B cells, such that B cells from different lymphoid compartments have unique carbohydrate composition on the LCA variable regions. One example is that the high expression of the CD45RB carbohydrate epitope recognized by antibody MT3 is restricted to B cells such as those in the splenic marginal zone. Another mab 6G3 also recognizes a CD45RB carbohydrate epitope which largely follows the reactivity pattern as MT3. Interestingly, an anti-CD45RA antibody KiB3 has a largely reciprocal reactivity pattern with MT3 such that it reacts with most B cells except for marginal zone B cells. Besides the marginal zone B cells from the spleen, a small proportion of B cells found in peripheral blood are also MT3/6G3 high and KiB3 low. Since marginal zone B cells have the immunophenotype of memory B cells [15], it is possible that the MT3/6G3 high B cells found outside the splenic marginal zone may represent circulating memory B cells. We hypothesized that high expression level of MT3/6G3 is a marker of memory B cells, similar to CD45RO being the marker of memory T cells. In this study we tested this hypothesis.

Methods and Materials

1. Peripheral Blood Mononuclear Cells

Umbilical cord blood samples were obtained from informed healthy volunteers immediately after normal vaginal deliveries at a local hospital. Pediatric peripheral venous blood samples were collected from afebrile patients who were admitted to the hospital for elective surgery; all of them had normal complete blood counts and differentials. Adult peripheral venous blood samples were received from healthy volunteers. Mononuclear cells were isolated from the blood samples using Ficoll-Paque (Pharmacia, Uppsala, Sweden).

2. Isolation of Tonsillar B Cell Subpopulations

Tonsils were obtained from routine tonsillectomy at a local hospital. Tonsillar lymphoid cells in suspension were first prepared by mincing the tonsil specimens in HBSS solution. Lymphoid cells from either tonsils or peripheral blood were then isolated with Ficoll-Paque (Pharmacia, Uppsala, Sweden) following manufacturer's protocol. For tonsillar cells, different lymphoid subpopulations were further separated with Percoll (Sigma). Cells harvested between Percoll gradient 60 and 80% were dense lymphocytes, highly depleted of germinal center B cells. Those harvested between 60 to 70% were enriched for MT3/6G3 high B cells, and those harvested between 70 to 80 % were enriched for MT3/6G3 low B cells. Both cell subpopulations were then purified by depleting T cells as follows: cells were first incubated with anti-CD2 and anti-CD3. After three washings with PBS (pH 7.5), Dyanbeads M-450 coated with sheep anti-mouse IgG (Dynal, Norway) were added to the cell suspension. After removing cells bound by magnetic beads, both cell subpopulations were >98% B cells. The resulting B cell subpopulations were between 80-90% enriched for MT3/6G3 high (percoll 60-70%) and 75 to 85% enriched for MT3/6G3 low B cells (percoll 70-80%).

3. Monoclonal Antibodies

Anti-CD45RA Mab's MB1 and MT2, anti-CD45RB Mab MT3 and 6G3 were produced in our laboratory. The anti-CD45RB identity of both Mab's were previously established by using LCA transfectants. Both Mab's MT3 and 6G3 recognize carbohydrate epitopes on CD45RB and they are largely coexpressed on the same cell populations. As 6G3 but not MT3 could be readily biotinylated, we employed 6G3 instead of MT3 for immunofluorescence stainings (see below). KiB3 (anti-CD45RA) and PD7/26 (anti-CD45RB) were kindly provided by Dr. Parwaresch (Germany) and Dr. Mason (UK) respectively. Supernatants of anti-CD21 (Bly-4) were produced in our laboratory. Mouse anti-human IgD, IgG, IgM, IgE, IgA, CD23 and Leu8 Mab's were obtained from Becton Dickinson. Mouse anti-human Mab Ki67, a proliferating cell marker, was purchased from Dakopatts, Denmark. Anti-CD11a Mab was obtained from AMAC,Inc. Westbrook, ME. Supernatants of anti-CD3 and CD2 were produced in our laboratory and the cell lines were obtained from the American Type Culture Collection, Rockville, Maryland.

4. Immunofluorescence stainings and flow cytometry

Isolated mononuclear cells were stained with three-color immunofluorescence technique and analysed with FACScan (Becton Dickinson). After the treatment of primary mabs at 4 C for 30 minutes, goat anti-mouse Mab, coupled with PE (Becton Dickinson), was applied for another 30 minutes at 4 C. After incubating with mouse serum for 15 minutes, the cells were exposed to biotinylated 6G3 followed by avidin-FITC (Becton Dickinson). Cells were finally stained with anti-CD20 conjugated with PercP. Three washings with phosphate-buffered saline (pH 7.5) were used between steps.

5. Cell culture Condition

The MT3/6G3 high and low B cell subsets obtained above were separately placed at a final concentration of approximately 1 million cells/mL in media consisting of RPMI 1640 supplemented with 20% FCS, L-glutamine (2 mM), penicillin (50 units/mL) and streptomycin (50 ug/mL) (Gibco, Grand Island, NY) in 50 mL culture flasks (Costar, Carnbridge). To each culture, phorbol 12-myristate 13 acetate (PMA) (Sigma chemical, St.Louis, MO) at a final concentration of 10 ng/mL, was added. Cytospins were prepared from the cells after culturing between 18 hours to two days.

6. Immunoperoxidase stainings

Three-micron thick B5-fixed paraffin-embedded tissue sections were deparaffinized through xylene and graded alcohols. The slides were incubated with 1:10 diluted supernatants for 30 minutes, followed by a second step incubation with peroxidase-conjugated rabbit anti-mouse Ig antibodies (Jackson Laboratory), diluted 1:50, for 30 minutes. Peroxidase activity was visualized with 3-amino-9-ethylcarbazole and H2O2 for 10 minutes.

Results

1. Tissue Distribution of MT3/6G3 high B cells

In addition to the splenic B cells, $MT3^{high}$ B cells could also be found in the other lymphoid organs. In tonsils, there were clusters of $MT3^{high}$ B cells localized in the subepithelial layer. The germinal center, interfollicular and mantle zone B cells were largely negative with MT3 staining. However, a proportion of the mantle zone B cells (roughly 30%) were positive with MT3. Mab 6G3 followed a similar reactivity pattern as MT3.

In peripheral blood, the expression of MT3 was also parallel to that of 6G3 (Fig. 1). Around 25% of peripheral B cells were $MT3/6G3^{high}$ in adults (Fig. 2). In contrast, B cells in cord blood showed only a single population and uniformly $MT3/6G3^{low}$ (fig. 2). The distinction of $MT3/6G3^{dim}$ and $MT3/6G3^{high}$ B subsets was first detected at around 9 months of age. The percentage of $MT3/6G3^{high}$ cells also increased with age, with 10% at the age of 9 months, to 15% at 2 years and 25% at 27 years (not shown).

2. Characterization of MT3/6G3 high cells

Histologically, MT3/6G3 high B cells found in tonsils were similar to those in marginal zone of spleen, with bigger cell size and more cytoplasm than those in mantle zone. When tonsillar B cells separated based on the cell density using percoll centrifugation, cells harvested between 60 and 70% were enriched for $MT3/6G3^{high}$ (80-90% enriched) whereas cells harvested between 70 and 80% were enriched for $MT3/6G3^{dim}$ (75-85% enriched) (fig.3).

We then analyzed the immunophenotype of the these two B subsets using flow cytometric technique, and the results were summarized in Table 1. Fig.4 showed that a significant proportion of MT3/6G3^{high} B cells did not express surface IgD or IgM, suggesting that these cells have encountered antigens and undergone immunoglobulin switching. In contrast, MT3/6G3^{low} B cells largely coexpressed surface IgM and IgD, reflecting their antigenic-inexperienced status. Furthermore, MT3/6G3^{low} B cells but not MT3/6G3^{high} B cells expressed CD23, a marker for virginal B cells. Leu-8, the human homologue of Mel-14 which is a homing receptor to the peripheral lymphoid organs in the murine system, was homogenously expressed on MT3/6G3^{low} B cells but only a subset of MT3/6G3^{high} B cells. This finding implies that MT3/6G3^{high} B cells have more diversified functions and are required to circulate to different anatomic sites. Peripheral blood MT3/6G3^{high} B cells, as well as splenic marginal zone B cells, had similar immunophenotype as tonsillar MT3/6G3^{high} B cells.

3. Responsiveness to stimuation in vitro

Upon stimulation by TPA, MT3/6G3^{high} B cells had an early response, as detected by Ki-67 positivity 2 days post-stimulation (up to 40%). Furthermore, MT3/6G3^{high} B cells also demonstrated strong aggregation and clumping detected as early as 18 hours post-stimulation. In contrast, Ki67 expression was found only in a low proportion of MT3/6G3^{low} B cells (10%) at two days. In addition, cell aggregation at 18 hours after being stimulated by PMA was much weaker than that of MT3/6G3^{low} cells (Fig. 5).

Discussion

These results showed that the expression level of two CD45RB epitopes detected by MT3 and 6G3 allows the distinction of two functional B subsets. In addition to the splenic marginal zone, the MT3/6G3^{high} B cells can be found in peripheral blood and the subepithelial layer of tonsils. On the basis of the immunophenotype, they likely belong to the same cell type and represent the counterparts of the splenic marginal zone B cells. MT3/6G3^{high} B cells as a group are antigen experienced (ie. memory B cells), as shown by their gradual increase in proportion in postnatal life, immunophenotype and early responsiveness to mitogen in vitro.

The absence of MT3/6G3^{high} B cells in the cord blood suggests that this B subset is likely the product of antigenic challenge and is developed only postnatally. In fact, it is known that splenic marginal zone cells respond rapidly to certain antigens (polysaccharides), and it has been suggested that the lack of the splenic marginal zone contributes to the hypo-responsiveness to these antigens in children under the age of two. This phenomenon supports the notion that MT3/6G3^{high} B cells play a crucial role in the immune system. Interestingly, our results showed that the age at which MT3/6G3^{high} B cells can be first detected is around 9 months, preceding the development of splenic marginal zone at two years of age [16]. It is tempting to speculate that some of the MT3/6G3^{high} B cells generated by antigenic challenge will circulate to the spleen and colonize around the mantle zone, thereby creating the marginal zone.

MT3/6G3^{high} B cells found in the subepithelial layer of tonsils may also be derived in the same way as the splenic marginal zone. The localization of these memory B cells in the subepithelial layer, a site which is in close proximity to the external environment, can promote mounting a rapid and efficent immune response. Consistent with this concept, a significant proportion of MT3/6G3^{high} B cells do not express Leu8 antigen, and therefore these cells are able to circulate different anatomic sites other than peripheral lymph nodes.

In summary, we used the expression level of CD45RB detected by mab's MT3 and 6G3 to define two distinct B subsets. Although MT3/6G3^{high} B cells can be found in different anatomic sites, they are related to each other by a relatively uniform imunophenotype. As a group, they represent memory B cells which can be disseminated to specialized regions such as the marginal zone of spleens and subepithelial layer of tonsils. In contrast, MT3/6G3^{low} B cells represent virginal B cells constantly replenished by the bone marrow. Based on these results, a model of B cell differentiation is illustrated in figure 6.

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Table 1. Immunophenotypes of MT3 high vs MT3 low B cells

		MT3 high	**MT3 low**
1.	sIgM	+	+
2.	sIgD	+/-	+
3.	sIgA/E/G	+	-
4.	CD23	low	high
5.	Leu8	high/low	high

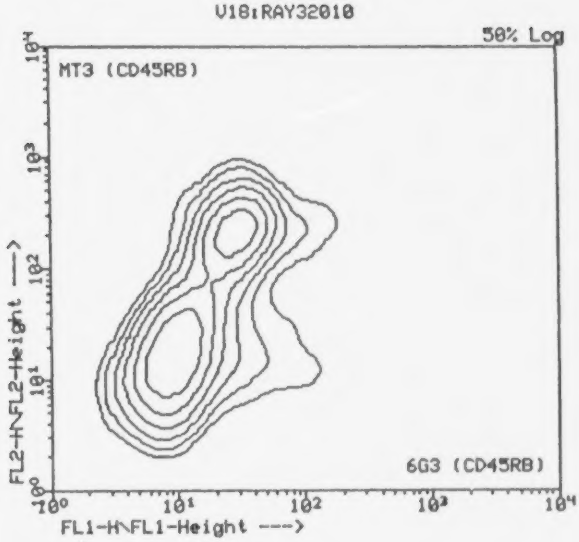


Fig.1
The expression of MT3 is directly proportional to that of 6G3 within the B cell population.

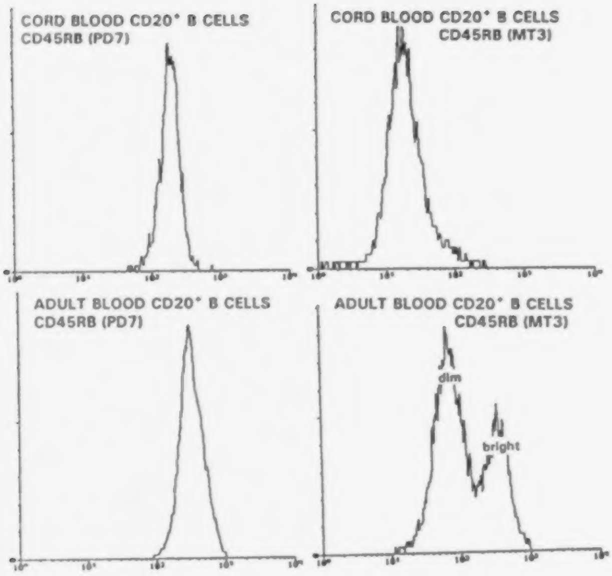


Fig.2
FACScan analysis of cord blood CD20$^+$ B cells (upper row) and peripheral blood CD20$^+$ B cells (lower row) with CD45RB reagents (left: PD7/26; right: MT3). Cord blood B cells show strong uniform staining for PD7/26 and weaker staining for MT3. Adult blood B cells have slightly stronger PD7/26 staining and show a bimodal staining pattern in which both the MT3^{dim} and MT3^{bright} populations stain stronger than the cord blood B cells.

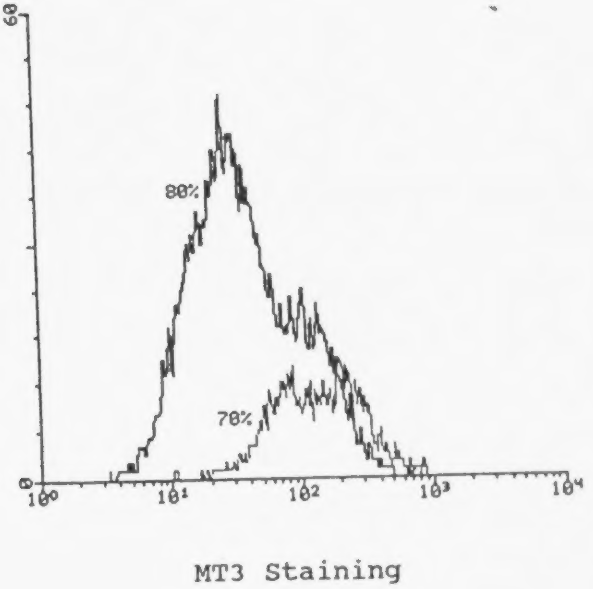
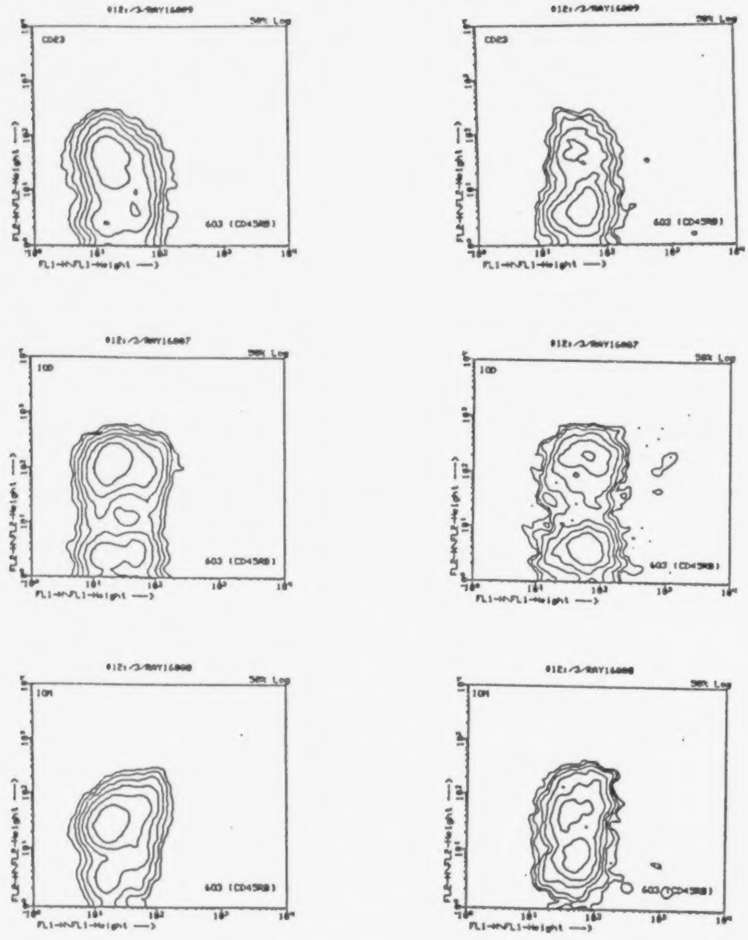
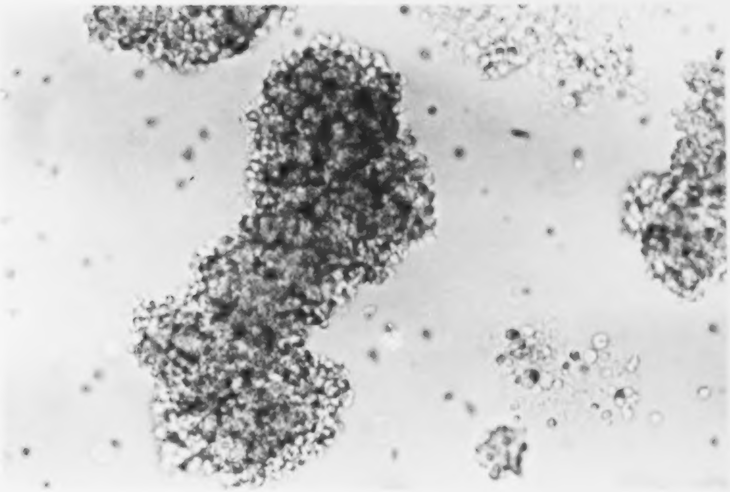


Fig.3
Separation of tonsillar B cells into two subpopulations using percoll gradient. Cells harvested between the 60 and 70% (labeled 70%) are uniformly $MT3^{high}$, whereas only a small proportion (about 20%) of those cell harvested between the 70 and 80% (labelled 80%) are $MT3^{high}$.

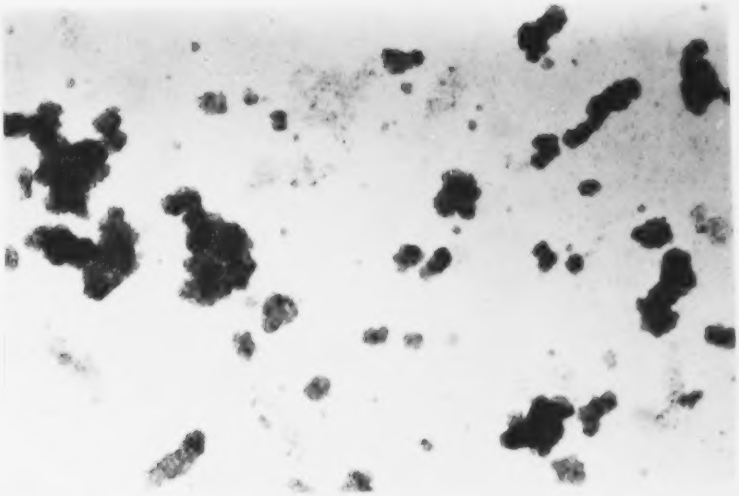


MT3 low MT3 high

Fig.4
Phenotypic analysis of MT3/6G3 low (left column) and low (right column) B cells. Note that MT3^{low} B cells are largely CD23, IgD and IgM positive. In contrast, MT3^{high} B cells do not express CD23 and only about 50% of them express IgD or IgM.



a. MT3^{high} B cells



b. MT3^{low} B cells

Fig.5
After incubating with phorbol ester for 24 hours, MT3^{high} B cells (a) showed much more aggregation than MT3^{low} B cells (b).

Bone Marrow ---->

antigen
Virginal B cells ---------->

Germinal Center ------->

Memory B cells ---->

1) marginal zone
2) sub-epithelial
layer of tonsils
3) peripheral blood

MT3 Expression

0 --------------> dim ---> bright --------->

Figure 6. Scheme of B cell Differentiation

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

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1. Diversity of LCA Expression

Using a panel of monoclonal antibodies (mab's) reactive with either protein- or carbohydrate-associated epitopes present on the LCA extracellular domain, we were able to demonstrate LCA structural variations which are far more extensive than previously known.

1a) Heterogeneity of LCA Extracellular Domain

It is clear that differential splicing of mRNA generates heterogeneity, but only to a maximum of eight possible isoforms. Until recently, only five LCA isoforms were actually known to exist, including ABC (220 kd), AB and BC (205 kd), B (190 kd), and O (180 kd). Dianzani et al [1] provided the first evidence for the existence of one additional LCA isoform, C (190 kd) which is expressed on peripheral T cells. During our study of CD45RC (chapter 6), we obtained results which supported the existence of LCA isoform C and also showed another additional LCA isoform, namely AC (205 kd) which appears to be confined to the B-cell lineage. Therefore, a total of seven human LCA isoforms are known to date.

The LCA extracellular domain, in particular the variable region, is heavily glycosylated [2]. Our study showed that these carbohydrates are differentially expressed in a cell type-specific manner. The most illustrative example is from examining the tissue distribution of CD45RA epitopes in various B-cell anatomical compartments, including the mantle zones, marginal zones and germinal centers (Chapter 1, Table 3). Since virtually all B cells express the variable region A predominantly in the form of the ABC isoform (220 kd), heterogeneous CD45RA expression can be attributed to differential glycosylation. With the same approach, we demonstrated differential glycosylation in the variable regions B, C as well as the common region (CD45) in the B-cell lineages.

Compared to B cells, T cells utilize alternate splicing of mRNA to generate LCA variation to a greater extent. During T cell differentiation, there is a cyclic change of LCA expression between those of higher MW (220 and 205 kd) and those of lower MW (190 and 180 kd). Within the thymus, LCA expression starts with high MW isoforms on thymic progenitors, switching to those of low MW on immature thymocytes, and again changing to those of higher MW on mature thymocytes [3]. In the periphery, virginal T cells deriving from the mature thymocytes (with high MW LCA species) re-express LCA isoforms of lower MW upon antigenic stimulation [4,5].

I addition, differential glycosylation can be clearly demonstrated in T cells. One example is from the observation that the KiB3 epitope (CD45RA) is expressed in a proportion of CD45RA$^+$ T cells in postnatal but not neonatal blood samples (chapter 7). To explain this change in the variable region A, we first formulated the topographic relationships among the three CD45RA epitopes studied: namely MB1, MT2 and KiB3 (fig. 1a,b,c), on the basis of the results obtained in chapter 2. As the KiB3 epitope is usually covered by O-linked sugars on the variable region A, this suggests that modification of the carbohydrates in the variable region A must occur before the KiB3 epitope can be exposed.

Examination of LCA expression in peripheral monocytes also revealed differential glycosylation of the variable region O, as shown in the comparative study of two anti-CD45RO Mab's UCHL-1 and OPD4 (chapter 5). Therefore, differential glycosylation is universally found in all cell types examined in this study. Moreover, this phenomenon involves all the variable regions, as well as the common region (chapter 2).

1b. Differential Glycosylation of LCA - not a random process

In addition to the cell-type specificity, there is other evidence suggesting that differential glycosylation of LCA is not a random process.

First, there are certain associations between individual LCA isoforms and the expression of specific glycosylations. For instance, although both the MT2 and MB1 epitopes can be expressed by the isoform ABC (220 kd), only the MT2 but not the MB1 epitope is found on the AB isoform (205 kd). As the MT2 epitope can block the MB1 epitope (fig. 1), it is possible that the AB isoform is associated with a higher sialylation state on the variable region A, leading to the expression of MT2 but not that of MB1. In fact, MB1 became reactive with the 205 kd (AB) isoform after neuraminidase treatment causing desialylation (chapter 2). Another example is the expression of the sialic acid-dependent CD45RB epitope MT3 on T cells. Unlike the other anti-CD45RB mab's, MT3 recognizes predominantly the isoform B (190 kd). This suggests that the expression of the MT3 epitope is largely inhibited by the presence of the variable regions A and C.

The second line of evidence comes from studying the tissue distribution of the MT3 epitope which is largely reciprocal to the KiB3 epitope (CD45RA) within the B-cell lineage. The MT3$^+$KiB3$^-$ phenotype can be seen in the marginal zone of spleen. Within this B-cell subset, it is perceivable that, in all LCA isoforms carrying both variable region A and B (ie. ABC and AB), the expression of the MT3 epitope is associated with a glycosylated state in the variable region A, leading to a cover up of the KiB3 epitope (Fig. 2a). On the contrary, the MT3$^-$ KiB3$^+$ phenotype which can be seen in the mantle zone of lymphoid follicles, requires exposure of the KiB3 epitope as well as hypo-sialylation (at least focally) of the variable region B, leading to loss of expression of the MT3 epitope (Fig. 2b). Therefore, there is a tight coordination between the glycosylation process of variable regions A and B.

The third line of evidence is that many of these specific glycosylations do not exist in cord blood lymphocytes and thus may represent products of antigenic challenge. The emergence of these glycosylations at different ages also points to a regulated instead of a random process (chapter 7).

1c. Variants of LCA Isoforms

Differential glycosylation generates structural variants of LCA isoforms. For instance, CD45RA$^+$ T cells can be divided into KiB3$^+$ and KiB3$^-$ subsets, and both subsets are MT2$^+$. Knowing that the expression of the MT2 epitope is associated with the blockage of the KiB3 epitope (fig. 1), this implies that, within the KiB3$^+$ T-cell subset, there are at least two variants of the isoform ABC: one that have lost the MT2 epitope such that the KiB3 epitope can be exposed and one in which the ABC isoform retains the MT2 epitope and does not express the KiB3 epitope. The co-expression of these two variants of the isoform ABC permits the MT2$^+$KiB3$^+$ phenotype.

Studying the expression of the variable region B also reveals variants of the isoform B (190 kd). Within the CD45RO$^+$ T cells, mab's such as MT3 and 6G3 can distinguish two subpopulations which are CD45RB^{high} and CD45RB^{low}. As the isoform B is the predominant LCA species carrying the variable region B in CD45RO$^+$ T cells, and since the level of CD45RB expression remains fairly constant, the difference in CD45RB expression suggests the existence of at least two variants of the isoform B: one with MT3/6G3 epitope and the other one without. The relative abundance of these two variants expressed by a cell then determines the level of MT3/6G3 expression. Importantly, as shown on the flow cytometer (chapter 4), these two subsets are very distinct, implying that the relative proportion of this two variants is not in a continuum.

While the above two examples focus on individual variable regions for simplicity, it can be imagined that combinations of variations in different LCA domains may exist, providing great diversity of LCA expression. Each of these LCA variants may have different functional roles (ie. different ligands) and a wide range of LCA expression profiles can thus be created.

1d. The Biological Significance of the Diversity of LCA Expression

Many of the variations we have demonstrated are associated with sialic acid, which is commonly found as the terminal sugar residue on the carbohydrates of glycoproteins. As sialic acids are negatively charged, their existence on lymphocyte cell surface may be to prevent nonspecific adhesion such that cells can remain in the circulatory state, maximizing the chance of meeting the appropriate antigens. As LCA contributes to a high proportion of the cell-surface carbohydrates on lymphoid cells, hypo-sialylation of LCA will lead to a significant decrease in negative charge and therefore allow cell-cell interaction. In the case of germinal centers where cell-cell interactions of B cells with dendritic reticulum cells and with macrophages are commonplace, there is in fact 'global' hypo-sialylation of LCA involving all three variable regions and also the common region. Further, hyposialylation of LCA on germinal center B cells exposes peanut lectin receptors (chapter 2) which allow protein-carbohydrate interactions.

Therefore, one aspect of the biological significance of the abundance of carbohydrates on LCA is to provide a framework on which the cell-surface electrical charge can be regulated. This will have significant impact to inter-cellular interactions if the modifications involve a large proportion of the surface sialic acid expressed on the cell surface such as germinal center B cells. However, most of the glycosylation modifications we observed involve only a small proportion of sialic acid such that only one or two sialic acid-dependent epitopes are lost. These changes may be sufficient to modify ligand-receptor interactions of a few LCA species on the same cell surface.

More recently, there are data from different laboratories to suggest that specific LCA isoforms are associated physically with other crucial accessory molecules on the cell surface of T lymphocytes. Using an antibody-induced co-capping technique, it has been shown that LFA-1 co-caps with $CD45RA^+$ isoforms, CD2 with $CD45RO^+$ isoform, and CD4 and CD8 with $CD45RC^+$ isoforms. With the chemical cross-linking studies, however, CD2 is found to be associated with not only $CD45RO^+$, but rather with multiple LCA isoforms are found [7]. The $CD45RO^+$ isoform has also been reported to be associated with CD26 (dipeptidyl peptidase IV) [16]. Although these data may require further confirmation, they are consistent with the model that heterogeneity of the LCA extracellular domain is important in determining the exact LCA ligand(s).

1e. LCA Glycosylation

Differential glycosylations are post-translational events as they do not involve the LCA protein backbone. The control of these modifications is unknown. However, as discussed above, the control appears to be tightly controlled and linked to cell differentiation and development. It has been shown that LCA glycosylation occurs in the golgi apparatus and that the carbohydrate component is required for cell surface expression and stability of LCA. Therefore, impairment of carbohydrate incorporation by tunicamycin inhibits LCA expression, despite the fact that LCA in the precursor form can be detected inside the cytoplasm. Importantly, LCA in the non-glycosylated forms presumably has an immature conformation such that the tyrosine phosphatase activity is absent. Therefore, the function of LCA glycosylation is at least four-fold: 1) to create structural heterogeneity with the extracellular domain for ligand selection; 2) to provide stability of the molecule so that it can be expressed on the cell surface; and 3) to provide appropriate conformation allowing enzyme activity; 4) to regulate the overall cell surface negative charge.

2. LCA Expression and Lymphocyte Subsets

Analysis of LCA epitopes has allowed the delineation of a number of lymphoid subsets. Early studies separated memory T cells from virginal T cells on the basis of CD45RO and CD45RA expression respectively. CD45RO T cells can be divided into CD45RB^{high} and CD45RB^{low} [8] as confirmed by our studies which employed a number of different anti-CD45RB Mab's (chapter 4). Indirect evidence suggests that CD45RB^{high} T cells have TH1 activity (helper for Delayed-type Hypersensitivity reaction) whereas CD45RB^{low} T cells have TH2 activity (helper for Antibody production) [8,9].

Parallel to the reciprocal expression pattern of CD45RA and CD45RO in T-cell lineage, CD45RB MT3 and CD45RA KiB3 are mutually exclusive in B-cell lineage. Furthermore, high expression of MT3 (and KiB3^{low}) is a marker of memory B cells, with unique tissue distribution, immunophenotype and in-vitro activation responsiveness (Chapter 8).

Anti-CD45RA KiB3 also allows delineation of CD4^{+} CD45RA^{+} T cells into KiB3^{+} and KiB3^{-} subsets. Although the large majority of CD4^{+} CD45RA^{+} are KiB3^{-}, the proportion of KiB3^{+} appears to increase with donor's age. Furthermore, KiB3^{+} cells have elevated levels of LFA-1, a marker of memory cells. Taken together, KiB3^{+} CD45RA^{+} CD4^{+} cells may represent a group of antigen-experienced T cells which retain CD45RA+ isoforms.

In summary, studies of LCA variants allow delineation of lymphoid subsets not previously recognized. These lymphoid subsets appear to have distinct functional roles. This is consistent with the notion that expression of LCA is closely linked to the functional states of lymphoid cells.

3. Unsolved Issues

3a. Functional Roles of LCA

It is clear that the expression of LCA variants is tightly associated with lymphoid subsets with different functions, and that LCA has tyrosine phosphatase activity in the cytoplasmic domain. Whether the expression of a specific LCA pattern directly contributes to a specific functional phenotype remains unclear. A large number of in-vitro studies using anti-LCA antibodies to alter LCA conformation showed a wide range of biological effects but so far has not generated an unifying concept. Some of the results in fact appear to contradict each other. One explanation relates to the nature of the anti-CD45R Mab's employed in those studies. For instance, incubation with an anti CD45RA Mab may biologically alter all LCA variants carrying the variable region A (i.e. ABC, AB, AC), each of which can have different function(s). Therefore, the end result is the summation of all these biological effects which are presumably cell type- and antibody- specific.

3b. Ligands of LCA

As discussed, LCA is a family of a large number of related but distinct proteins differentially expressed on lymphoid cells. Each of these LCA variants likely has different ligand(s). Multiple approaches have been used to identify LCA ligands including antibody-mediated co-capping and chemical cross-linking experiments as discussed above. On the basis that an anti-CD45RO Mab can block the adhesion of isolated CD22 protein to T cells, Stamenkovic et al [10] concluded that CD22 is the ligand of CD45RO. However, the first two approaches lack specificity with anti-CD45R Mab's. Subsequent work has shown that CD22 in fact interacts rather non-specifically with a sialic acid-containing moiety found in many molecules other than CD45RO [11].

3c. Lymphoid Subsets delineated by LCA Expression and their Role in Diseases

LCA has been a useful marker for delineating lymphoid subsets. Changes in the frequency of these functional subsets have been reported in a number of different clinical disorders. For example, there is a selective loss of $CD4^+CD45RA^+$ cells and a reciprocal increase of $CD4^+CD45RO^+$ in patients with multiple sclerosis [12], systemic lupus erythematosus [13], and rheumatoid arthritis [14]. These changes could be the result of activation of virginal T cells in vivo and their accelerated transformation into memory T cells. Interestingly, patients with systemic lupus erythematosus develop auto-antibody (mostly IgM) reactive with LCA, preferentially those species with high molecular weight (220 and 205 kd). Therefore, cells with high levels of expression of the 220 and 205 kd species, namely CD8$^+$ and CD4$^+$CD45RA$^+$, may be selectively downregulated or eliminated from the circulation. Recent data suggest that the switch of TH1 function (cell-mediated response) to TH2 function (antibody-mediated response) may be the key event allowing HIV to proliferate and produce clinically evident immuno-suppression [15]. Monitoring of the frequency of the CD4+ CD45RB^{high} (TH1) and CD45RB^{low} (TH2) may be instrumental in assessing the severity of the infection. Finally, the use of anti-LCA Mab in renal transplantation in primates has been shown to improve graft survival; hence, selective modulation of LCA by Mab may be useful in providing fine-tuned immunosuppression in transplantation.

In summary, a better understanding of LCA expression and its function on different lymphoid subsets has clinical relevance. Anti-LCA Mab's define functionally important lymphoid subsets and can be useful in disease monitoring and intervention.

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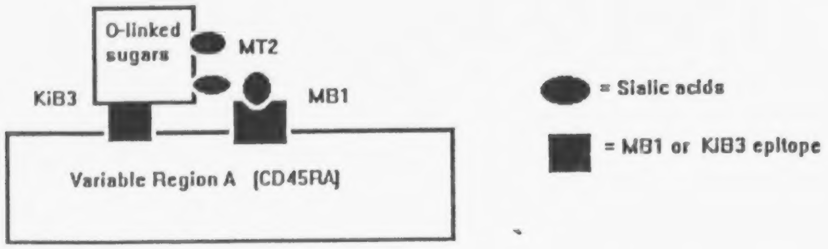
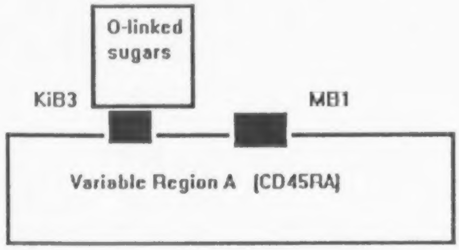
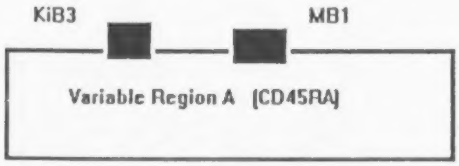

O-linked sugars
MT2
KiB3
MB1
= Sialic acids
= MB1 or KiB3 epitope
Variable Region A (CD45RA)

1a. The expression of the KiB3 and MB1 epitopes which lie on the protein backbone of the variable region A are covered by glycosylation. On top of this same group of glycosylation is the MT2 epitope which is made up of the terminal sialic acid-dependent sugar residues.

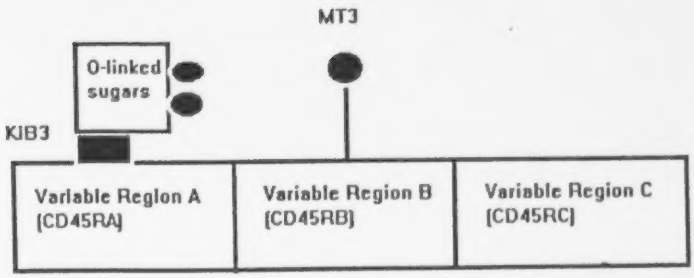

O-linked sugars
KiB3
MB1
Variable Region A (CD45RA)

1b. Desialylation causes the exposure of the MB1 epitope; The KiB3 epitope remains covered.

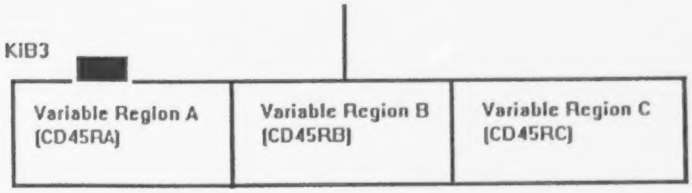

KiB3
MB1
Variable Region A (CD45RA)

1c. Removal of O-linked carbohydrates expose the KiB3 epitope.

Fig.1
Topographic relationships of the MB1, MT2 and KiB3 epitopes on the variable region A of LCA


MT3
O-linked sugars
KIB3
Variable Region A (CD45RA)
Variable Region B (CD45RB)
Variable Region C (CD45RC)

2a. The expression of the MT3 epitope (sialic acid-dependent) and coverage of the KiB3 epitope.


KIB3
Variable Region A (CD45RA)
Variable Region B (CD45RB)
Variable Region C (CD45RC)

2b. The expression of the KiB3 epitope and the loss of the MT3 epitope.

Fig.2
The mutually exclusive expression pattern of the MT3 (CD45RB) and KiB3 (CD45RA) epitopes